

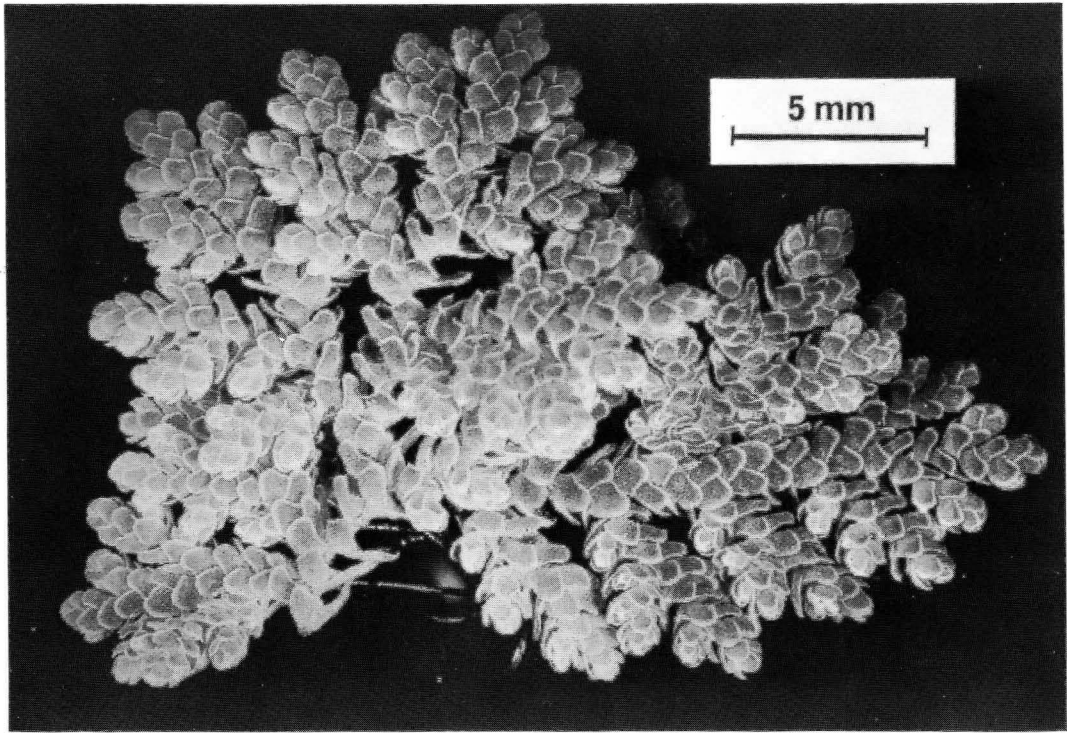
THE AUTECOLOGY OF *AZOLLA FILICULOIDES* LAMARCK  
WITH SPECIAL REFERENCE TO ITS OCCURRENCE IN  
THE HENDRIK VERWOERD DAM CATCHMENT AREA

Dissertation  
Submitted in Partial Fulfilment of the  
Requirements for the Degree of  
DOCTOR OF PHILOSOPHY  
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by

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FRONTISPIECE

Mature sporophyte of *Azolla filiculoides* Lam. showing general growth habit of branched rhizome and overlapping leaf lobes.

## ABSTRACT

An autecological study of the heterosporous fern *Azolla filiculoides* Lamarck and its endosymbiotic blue-green alga *Anabaena azollae* Strasburger, based on a combination of field and laboratory studies, is presented. The taxonomy, morphology and anatomy of the fern-alga association were studied as well as nutritional and physiological aspects of the symbiosis. These studies have defined the habitat and nutritional requirements of the fern and have provided new insights into its reproductive biology, nitrogen metabolism and the nature of the association between the fern and alga. In the catchment area of the Hendrik Verwoerd Dam the availability of suitably sheltered habitat limits the distribution of *A. filiculoides* while the availability of nutrients, in particular calcium, phosphorus and iron, limits the growth of the fern. The multilayered mats formed by *A. filiculoides* are essential for spore production, cause dramatic changes in the hydrochemistry of the underlying waters and confer a great competitive advantage on the plant. Methods for the isolation of the fern and algal components of the symbiosis have been developed but recombination of the individual organisms to reform the symbiosis was unsuccessful. The development of the fern is closely linked to that of the alga and the association is maintained throughout the life cycle of the fern. Because of its specific habitat and nutritional requirements, *A. filiculoides* is unlikely to colonize the open waters of the Hendrik Verwoerd Dam.

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## PREFACE

This dissertation describes the results of autecological studies of *Azolla filiculoides* Lam. with special emphasis on its growth potential in relation to the Hendrik Verwoerd Dam and its catchment area. These studies commenced in February 1971 and included both field and laboratory investigations of the fern-algal symbiosis and routine monitoring of *Azolla* populations and a series of environmental parameters at selected sites in the Hendrik Verwoerd Dam catchment area.

Chapter 1 contains a general introduction to the thesis and the background to the Orange River Development Project. Sampling techniques and the analytical methods which were used in this study are outlined in Chapter 2. The taxonomy and distribution of the genus *Azolla* in southern Africa are dealt with in Chapter 3, while Chapter 4 describes the results of morphological, anatomical and life history studies. The results of field and laboratory investigations on the environmental regulation of growth and nitrogen fixation are reported in Chapter 5 and the nutrient requirements of the symbiotic association are described in Chapter 6. Chapter 7 describes the isolation of the fern and algal components, and the pathways of nitrogen assimilation are characterized in Chapter 8. Except for Chapter 1 and Chapter 2, each chapter contains a review of the pertinent literature and is concluded by a discussion of its contents. These are brought together in a concluding discussion in Chapter 9, in which the results of the study are discussed in relation to each other and general conclusions are drawn as to the present and future growth of *A. filiculoides* in the Hendrik Verwoerd Dam catchment area. Finally, the main contents of each chapter are listed in an abstract.

Four appendices are attached to this thesis. The first describes the data used to calculate the minimum sample size, upon which the routine sampling programme was based. The second, third and fourth appendices comprise copies of papers that were published during the progress of the research and they are attached in chronological order. There is some unavoidable duplication in these papers because each was prepared for a different audience.

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## CHAPTER 1

### INTRODUCTION

The Orange River Development Programme (O.R.P.) was originally conceived purely as an irrigation scheme in the 1920s, but had to be shelved due to the lack of funds. However, rapid expansion in agriculture, industry and urban development during the 1940s and 1950s created an increasing requirement both for electricity and additional water supplies. This stimulated a re-evaluation of the project during 1960. At this stage a comprehensive development plan was drawn up to provide for the ultimate requirements of irrigation, urban water supply, industrial water supply, hydro-electric power development and flood control (Alexander, 1974). This concept widened the emphasis from the original irrigation scheme to the present multi-purpose Orange River Development Project, making it the most ambitious water management scheme undertaken in South Africa (Olivier, 1978).

The first phase entailed construction of the Hendrik Verwoerd Dam as a main water storage facility to regulate the flow of the Orange River and provide sufficient storage capacity for silt deposits. Water from the impoundment was introduced via a tunnel system into the catchments of the Great Fish and Sundays rivers in the eastern Cape Province. This was designed to relieve irrigation schemes affected by problems of mineralization and allow the development of new irrigation areas. The second phase, consisting of two further high diversion weirs, the Van Der Kloof and Torquay dams, with a network of canals supplying irrigation water to suitable areas of the lower Orange River valley (Jordaan, 1964; Kriel, 1971) is under construction.

In the planning stage it was envisaged that construction would begin during the 1960s and take place in stages over a number of years, with completion scheduled for the early 1990s (Jordaan, 1962). Final parliamentary approval for the project was granted in 1962 and the first phase of construction at the Hendrik Verwoerd Dam was initiated in 1966 (Olivier, 1978). Water storage began in September 1970 and, at the completion of construction in July 1971, the impoundment already contained 65 % of its full supply capacity (Kriel, 1971).

During the final planning stage prior to construction of the Hendrik Verwoerd Dam, the O.R.P. advisory committee recognized that the construction of an impoundment for irrigation and/or hydro-electric purposes would not only stabilize the hydrological regime but would also create a variety of new habitats for the aquatic biota (Naude, 1969). Where similar projects have been undertaken in other countries, newly-created impoundments have often caused serious problems. These have included the spread of disease vectors, dramatic changes in water chemistry and the development of excessive growths of aquatic macrophytes (Sculthorpe, 1967; Van Donselaar, 1968; Little, 1969; Mitchell, 1969, 1970, 1974). Despite obvious similarities in the types of problems encountered in different situations, the extent and severity of each problem depended on the specific organisms and conditions prevailing at each site (Lowe-McConnel, 1966; Obeng, 1969). Because of these features, it was felt that the available local knowledge was insufficient to fully predict the possible biotic and abiotic consequences of impounding the Orange River with any degree of certainty (Naude, 1969).

The O.R.P. advisory committee therefore recommended that a long-term programme of pre- and post-impoundment hydrochemical and hydrobiological research should be undertaken so as to provide the information necessary for successful implementation and management of the O.R.P. scheme. An inter-disciplinary research programme involving scientists from several research institutions was therefore initiated in 1968 (Noble and Botha, 1970).

Particular concern was expressed over the possibility that the impounded waters of the Hendrik Verwoerd Dam might also be colonized by aquatic macrophytes since one of the major problems often associated with newly-constructed impoundments has been the explosive growth of free-floating aquatic macrophytes (Little, 1966). Several pre-impoundment surveys of aquatic macrophytes were therefore conducted in the Hendrik Verwoerd Dam catchment area (Noble and Botha, 1970). These revealed the presence of a few species of submerged macrophytes (*Potamogeton* spp.) and one species of the free-floating symbiotic water fern *Azolla* (Edwards and Nel, 1972). The committee felt that the latter might be able to colonize the waters

of the Hendrik Verwoerd Dam because *Azolla* can fix atmospheric nitrogen via its blue-green algal symbiont (Moore, 1969). The plant is capable of colonizing nitrogen-deficient waters and has a history of rapid spread in other countries (Sculthorpe, 1967). It was thus recommended that an autecological study of *Azolla* be initiated, with particular emphasis on its growth potential in the Hendrik Verwoerd Dam and the associated catchment area. This study was to be carried out under the auspices of the O.R.P. working committee for hydrochemistry and hydrobiology and following a preliminary planning period the *Azolla* research programme commenced in February 1971.

The overall aims of the project were to assess the possibility that *Azolla* plants might colonize the impounded waters of the Hendrik Verwoerd Dam and, by means of autecological studies, characterize the growth potential of the fern-alga association. This project is effectively the basis of this thesis.

*Azolla* populations in the catchment area were routinely monitored to determine possible distributional changes while chemical analyses were used to monitor nutrient availability. Laboratory studies formed a large part of the research programme, but special emphasis was placed on the applicability of laboratory results to field conditions. The research programme included taxonomic identification of the *Azolla* material as well as morphological, anatomical and life history studies as an essential background for the ecological research. Particular attention was paid to the plant's nutritional requirements and its ability to tolerate a wide range of environmental parameters. The isolation of the fern and algal components formed an integral part of the research aimed at obtaining a better understanding of nitrogen assimilation by the symbiotic association, in particular the fixation of atmospheric nitrogen.

## CHAPTER 2

### MATERIALS AND METHODS

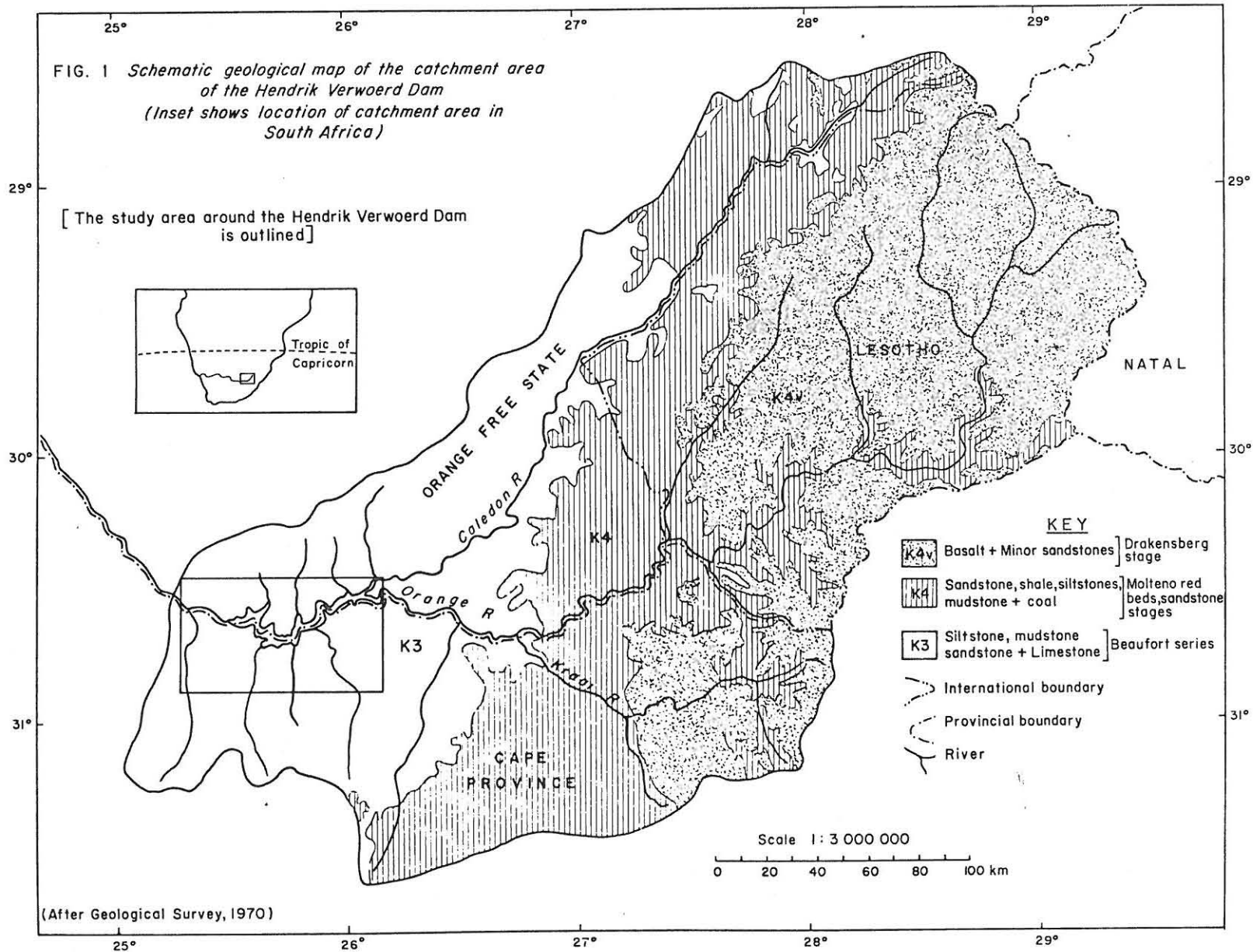
#### 2.1 THE STUDY AREA

##### 2.1.1 The Hendrik Verwoerd Dam and its catchment area

The Hendrik Verwoerd Dam is the largest impoundment in the Republic of South Africa. The dam wall is 85 m high and 905 m long, situated at latitude  $30^{\circ} 37' 30''\text{S}$  and longitude  $25^{\circ} 30' 15''\text{E}$ , 1 259 m above mean sea level (Olivier, 1978). At full supply level, the impounded water stretches 85 km eastward along the old river course to the confluence of the Orange and Caledon Rivers and extends a further 25 km up the Caledon valley and 32 km up the Orange River valley. At this level, the lake has an average width of 3,6 km, contains  $5\,952 \times 10^6 \text{ m}^3$  of water with a surface area of  $364,88 \text{ km}^2$  and has a surface area to depth ratio of  $404 \text{ ha m}^{-1}$  (Schutte and Bosman, 1972).

The catchment of the Hendrik Verwoerd Dam covers an area of approximately 70 642 square kilometres, lying between latitudes  $28^{\circ} 30'\text{S}$  to  $31^{\circ} 35'\text{S}$  and longitudes  $25^{\circ} 05'\text{E}$  to  $29^{\circ} 25'\text{E}$  (Figure 1). Within this area lies the Republic of Lesotho, portions of two South African provinces, namely the Cape Province and the Orange Free State, and a very small portion of the Republic of Transkei. The catchment area varies in altitude from 3 500 m around the source of the Orange River at Mont Aux Sources in the Drakensberg Mountains to 1 259 m at the Hendrik Verwoerd Dam. The Orange River, the major tributary of the Hendrik Verwoerd Dam, is approximately 520 km long from its source to its point of entry into the dam, with an average gradient of  $4,25 \text{ m km}^{-1}$ . The climate of the catchment area varies from the temperate, moist, cool alpine highlands of Lesotho westwards through increasingly more arid conditions to the dry karroid plateau around the Hendrik Verwoerd Dam (Edwards, 1974).

The geology of the catchment area is relatively simple, consisting of three basic stages, all of which form part of the Mesozoic Karoo System (Figure 1). The Drakensberg Stage (K4V) and the Molteno Red Beds and

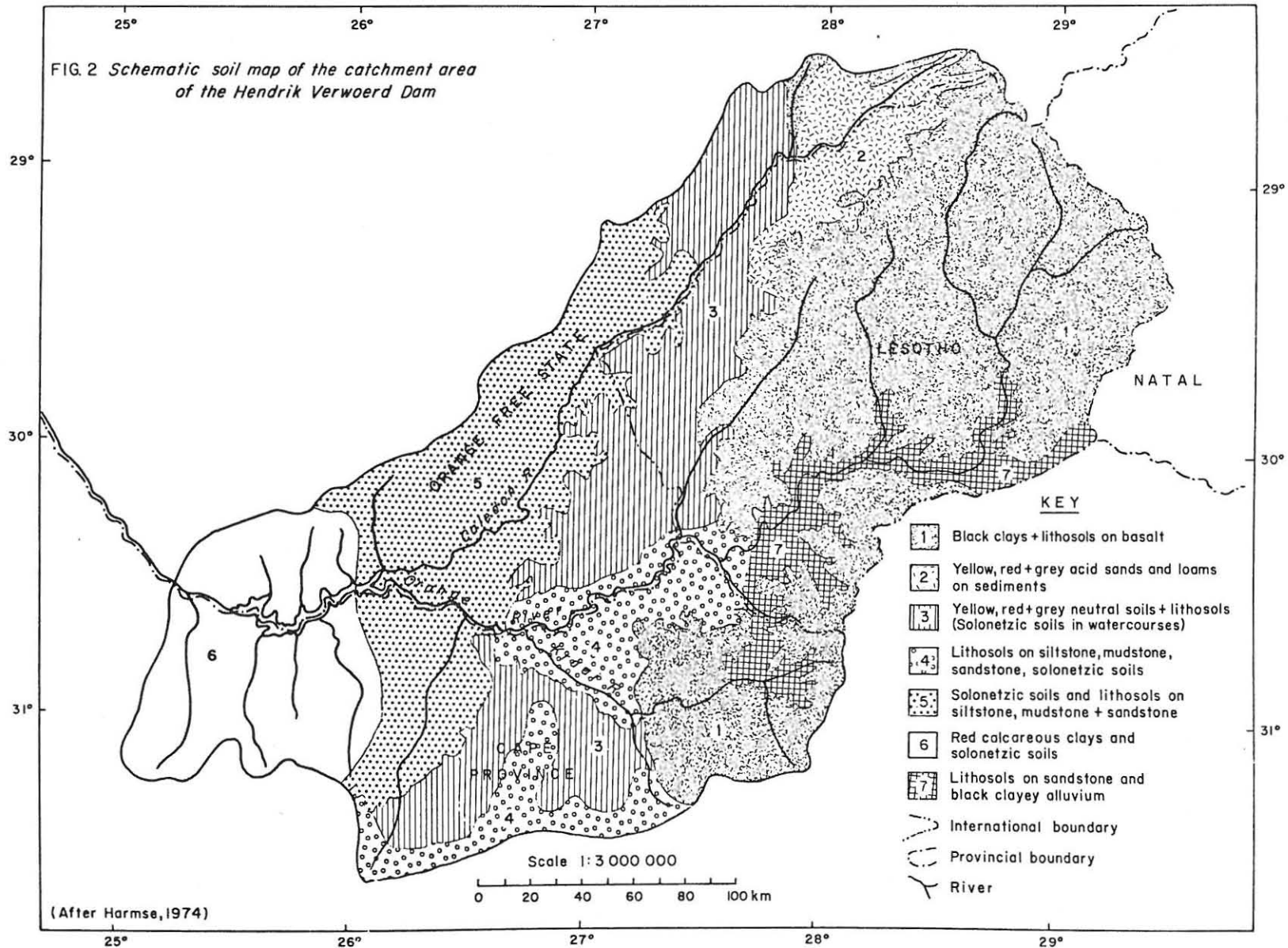


Cave Sandstones (K4) form part of the Stormberg Series, while the third stage (K3) forms part of the Beaufort Series (Geological Survey, 1970). The basalt and minor sandstones of the Drakensberg Stage are relatively resistant to weathering and form the tops of steep retreating scarps. The silt-rich mudstones, sandstones and siltstones of the Molteno Red Beds and Cave Sandstones become exposed on the steep slopes of the retreating escarpment. Underlying these two stages are the sedimentary sandstones, mudstones and siltstones of the Upper Beaufort Series.

Figure 2 shows a schematic soil map of the catchment area (adapted from Harmse, 1974). The influence of climate on weathering and soil formation in the catchment area is shown in the regional differences between the soils on similar units of landscape and parent rocks. There is a general decrease in rainfall from the north-east to the south-west and the soils in the drier south-west, on the same parent material, have a higher silt content than those in the north-east. The soils on the sedimentary rocks in the north-east are mostly neutral or acid, whilst the soils in the south-west are mostly solonetzic. Solonetzic soils are usually greyish or reddish sandy loams, acid in the A and alkaline in the darker clayey B horizon, and contain carbonates of lime and soluble salts (Van der Merwe, 1941). The younger soils on the upper slopes of the pediments and along the water courses are, however, also solonetzic in the wetter north-east (Harmse, 1974).

Three main perennial rivers flow into the Hendrik Verwoerd Dam. These are in order of importance, the Orange River, Caledon River and Kraai River. Several other small rivers and streams flow directly into the lake from the north and south (Figure 1).

Both the Kraai River and the Orange River with its two main tributaries, the Mantsonyane and Seati Rivers, flow initially over the basaltic lavas of the Drakensberg Stage. These rocks, being mechanically stable, contribute little in the way of suspended or dissolved matter to the water, which is neutral to slightly alkaline. As these two rivers then flow over the mudstones and siltstones of the Molteno Stage followed by the Beaufort Series, there is a progressive increase in silt and dissolved



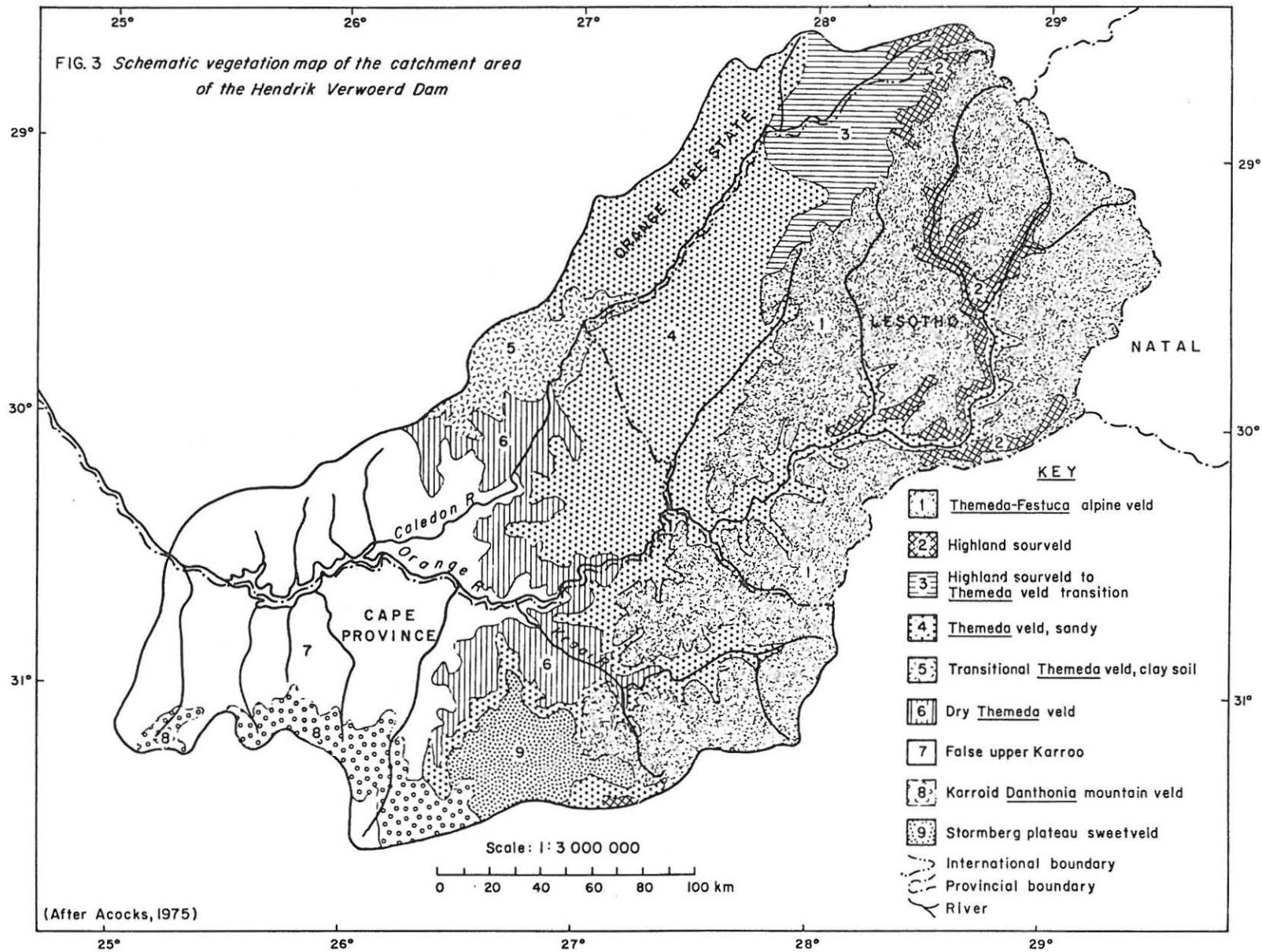
substances caused by leaching and erosion of these relatively unstable rock types (Keulder, 1974). The Orange River is, for the main part of its flow, in contact with the unstable mudstone of the Molteno Stage and Beaufort Series.

The Caledon River flows mostly over the mudstones and siltstones of the Molteno Stage and Beaufort Series. Due to the unstable nature of these rock types, they contribute significantly to the suspended and dissolved substances in the water. The Caledon River is thus a source of considerable amounts of silt and dissolved salts for the Hendrik Verwoerd Dam (Keulder, 1974).

The small, intermittently-flowing rivers to the north and south of the Hendrik Verwoerd Dam are of particular importance to this study, since it is in these rivers that large quantities of *Azolla* plants are found. In these rivers, flow rates are high during periods of summer rainfall but almost non-existent during the winter. For the whole of their lengths, these small rivers flow over the siltstones and mudstones of the Beaufort Series. Thus these rivers, when flowing, have high levels of silt and dissolved substances. In addition, the belts of limestone and dolerite found in this area contribute to the sodium, calcium and magnesium content of the water. During periods when the flow is low or non-existent, the belts of limestone and dolerite form natural dams across these small rivers, resulting in the formation of long pools, often 500 to 800 m in length.

The silt particles in the water from the catchment area have a high affinity for the adsorption of trace elements, particularly zinc and manganese (about 70 %), followed by iron and copper (about 50 %). Calcium and potassium are the most actively adsorbed among the macro-elements (about 45 % and 30 % respectively), while the silt has a relatively low affinity for sodium and magnesium adsorption (20 % and 25 % respectively) (Keulder, 1974).

The catchment area of the Hendrik Verwoerd Dam can be divided into nine distinct terrestrial vegetation types (Figure 3) (Acocks, 1975). These occur on three main habitat types: riverine, well-drained lithosolic



hill-slopes and flat areas. The communities that make up these three main habitat-vegetation complexes form three parallel series ranging from mesic to xeric in accordance with the east to west climatic gradient and local soil conditions (Edwards, 1974). The general trend is from moist alpine grasslands, through progressively drier grassland associations on sandy soils to the floristically poor False Upper Karoo (Acocks, 1975).

Edwards and Nel (1972) investigated the distribution and occurrence of aquatic macrophytes in the catchment area of the Hendrik Verwoerd Dam outside of Lesotho. Van Zinderen Bakker (1955) and Jacot Guillarmod (1962, 1963, 1972) have described the vegetation of the bogs and sponges in Lesotho. Jacot Guillarmod (1972) noted that floating aquatic vascular plants were absent or very sparse in the lowlands of Lesotho although species of *Marsilea* and *Potamogeton* were found. Edwards and Nel (1972) found fifteen species of aquatic macrophytes, of which *Azolla* was the only significant free-floating aquatic species.

#### 2.1.2 Location of the study area and sampling sites

During an initial survey, 478 localities inside the catchment area were examined for the presence of *Azolla* plants. Subsequently, this survey was extended to include 81 additional localities in the area downstream from the dam wall (incorporating the Oorlogspoort River), since large quantities of *Azolla* plants had been seen in this area.

The fern was found to occur in only 57 localities. These were small farm dams and riverine sites in the area immediately north, south and south-west of the Hendrik Verwoerd Dam. No evidence of the presence of *Azolla* plants was found in either the Caledon, Orange or Kraai Rivers.

The study area was therefore chosen to include all sites in or near the catchment area of the Hendrik Verwoerd Dam where *Azolla* plants had been found during the initial survey. Figure 1 shows the location of the study area within the catchment area of the Hendrik Verwoerd Dam. The study area covers an area of approximately 3 410 km<sup>2</sup>, lying between latitudes 30° 28'S to 30° 52'S and longitudes 25° 18'E and 26° 00'E. The vegetation is classified as False Upper Karoo (Acocks, 1975), growing on red calcareous

clays and solonchic soils (Harmse, 1974), derived from the siltstones, mudstones, limestone and dolerite of the Upper Beaufort Series (Geological Survey, 1970). Figure 4 shows the distribution of *Azolla* plants in the study area and the location of the twelve sampling sites. These sites were chosen, after the initial distribution survey, so as to cover the whole range of habitats occupied by the fern. The dimensions and exact location of each sampling site are given in Table 1, while plates 1 to 12 show the aspect and associated vegetation. [During the study period, sampling sites 1, 2 and 3 (see Figure 4) were ruined by the dredging of their respective streams for the construction of new bridges along the Bethulie-Oranjekrag road.]

The study area lies within the summer rainfall zone of South Africa and normally receives 300-340 mm of rain per year. However, during the study period (1971-74) the average annual rainfall was 545 mm due to the exceptionally heavy rains (910 mm) that fell during 1974. Figure 5 shows the monthly rainfall recorded at five towns in the study area from January 1971 to June 1974, while Figure 6 shows the variation of maximum, mean and minimum rainfall for the same data. The rainfall and temperature figures quoted here have been calculated from data published by the South African Weather Bureau (South African Weather Bureau, 1971-1974). Unfortunately, the weather stations near the Hendrik Verwoerd Dam do not record wind direction and strength or hours of sunlight.

The highest monthly rainfalls may be recorded during any of the months between January to April, usually with a short period of high rainfall in October, while minimum rainfall values are recorded during the months May to September. Rainfall in the study area is mainly of thunderstorm origin, so that large volumes of rain fall in short time intervals. Due to the flat terrain and the rather sparse vegetation cover, sheet erosion occurs in many areas. Flooding and scouring of rivers during the peak rainfall months of January to April have a marked effect on riverine aquatic plant life (Ashton, 1974).

Maximum and minimum air temperatures vary between 22,0 °C and -4,9 °C in winter and between 39,8 °C and 22,2 °C in summer. Winds, which are not seasonal, blow throughout the year, predominantly from the north and

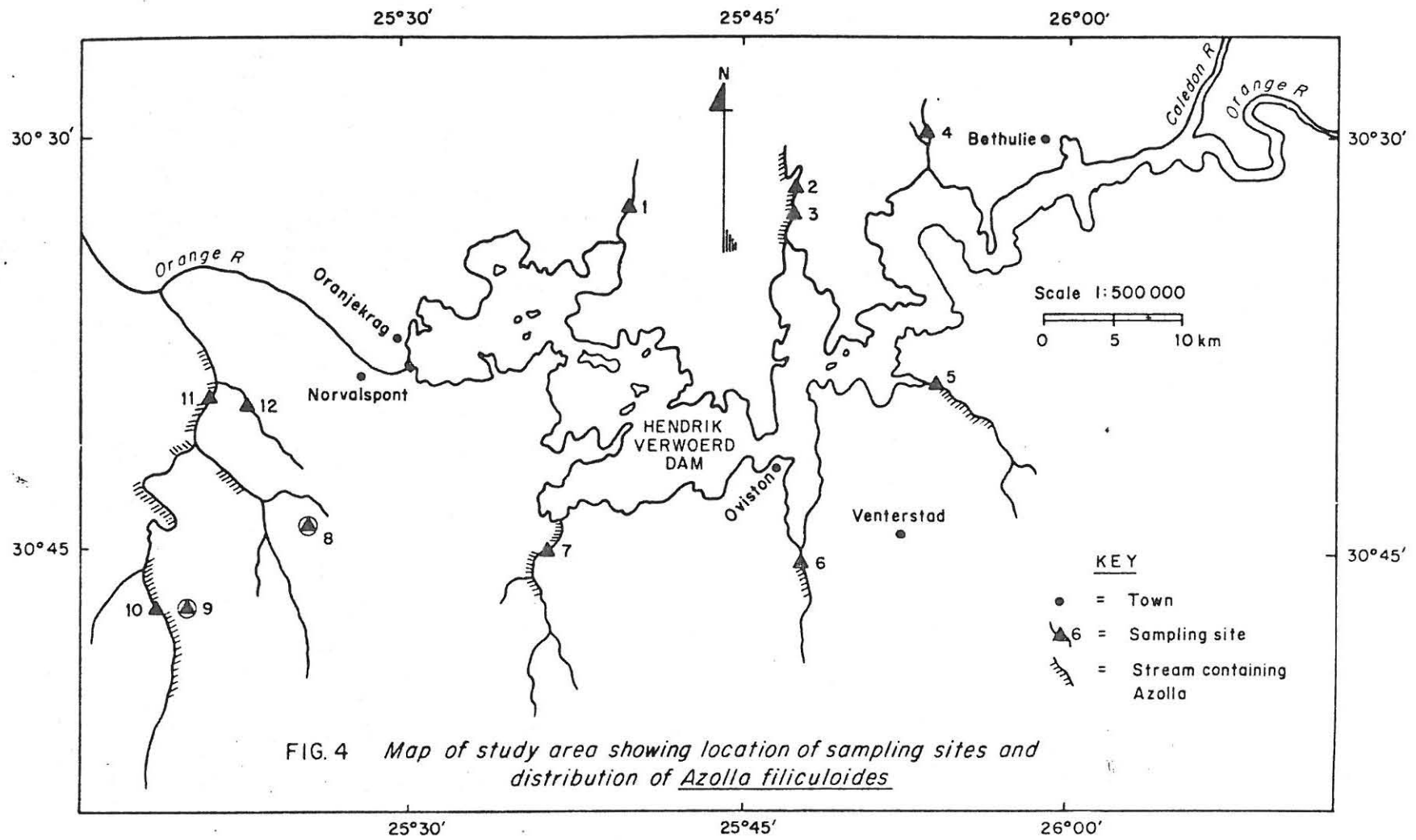


FIG. 4 Map of study area showing location of sampling sites and distribution of *Azolla filiculoides*

TABLE 1 - Grid references and areas of sampling sites in the study area shown in Figure 4

Station No.	Name	Altitude	Grid References	Dimensions	Area	Type
1	Kromberg	1 274 m	30° 32' 39"S 25° 40' 14"E	2 m x 60 m	120 m <sup>2</sup>	Stream
2	Bossiespruit North	1 279 m	30° 31' 13"S 25° 47' 57"E	8 m x 100 m	800 m <sup>2</sup>	Stream
3	Bossiespruit South	1 277 m	30° 31' 31"S 25° 47' 57"E	6 m x 100 m	600 m <sup>2</sup>	Stream
4	Zoetvlei	1 268 m	30° 30' 39"S 25° 53' 42"E	2 m x 50 m	100 m <sup>2</sup>	Stream
5	Broekpoortspruit	1 260 m	30° 39' 28"S 25° 53' 42"E	10 m x 100 m	1 000 m <sup>2</sup>	Stream
6	Brakspruit	1 265 m	30° 46' 31"S 25° 46' 54"E	6 m x 100 m	600 m <sup>2</sup>	Stream
7	Suurbergspruit	1 265 m	30° 44' 42"S 25° 36' 19"E	4 m x 100 m	400 m <sup>2</sup>	Stream
8	Ratelpoort	1 308 m	30° 44' 22"S 25° 25' 01"E	10 m diam.	78,5 m <sup>2</sup>	Reservoir
9	Kleintoren Dam	1 320 m	30° 46' 57"S 25° 19' 46"E	8 m x 100 m	800 m <sup>2</sup>	Reservoir
10	Kleintoren River	1 310 m	30° 46' 51"S 25° 19' 09"E	10 m x 100 m	1 000 m <sup>2</sup>	Stream
11	Reitfontein	1 213 m	30° 38' 04"S 25° 21' 30"E	8 m x 80 m	640 m <sup>2</sup>	Stream
12	Van Wyksfontein	1 245 m	30° 39' 32"S 25° 22' 34"E	6 m diam.	28,3 m <sup>2</sup>	Spring



PLATE 1: STATION 1 (Kromberg)

Small, non-perennial stream covered with mat of *A. filiculoides*. Marginal vegetation consists mainly of *Scirpus* sp., *Cyperus* sp. and *Eragrostis lehmanniana*, with some *Acacia karroo* (partly visible at upper left).



PLATE 2: STATION 2 (Bossiespruit North)

Large perennial stream covered with mat of *A. filiculoides*. Marginal vegetation consists of *Scirpus* sp. (foreground) and *Eragrostis lehmanniana*, with large specimens of *Acacia karroo* (upper right) and *Alnus* sp. (upper left) providing shade for marginal plants of *A. filiculoides*.

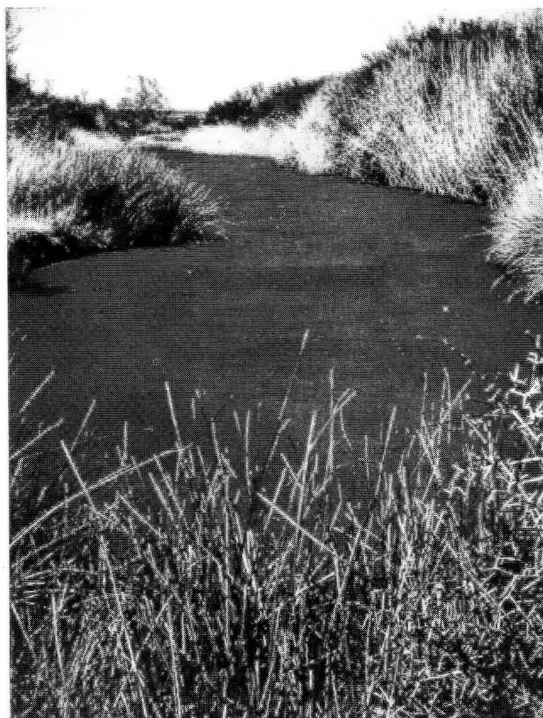


PLATE 3: STATION 3 (Bossiespruit South)

Large perennial stream covered with thick mat of *A. filiculoides*. Marginal vegetation dominated by *Scirpus* sp. (foreground and left) and *Phragmites* sp. (upper right), providing little or no shade.



PLATE 4: STATION 4 (Zoetvlei)

Small, non-perennial stream covered with mat of *A. filiculoides*. Marginal vegetation consists of a dense growth of *Phragmites* sp. (right) with scattered tussocks of *Eragrostis lehmanniana* and some *Alnus* sp. (left).



PLATE 5: STATION 5 (Broekpoortspruit)

Small perennial river, flowing through typical False Upper Karoo vegetation. Two limestone ridges at right angles to the river are seen in the foreground, resulting in pool formation. Marginal vegetation consists of some *Scirpus* sp. and *Eragrostis lehmanniana*. (No *A. filiculoides* present since photograph taken in March, after floods.)



PLATE 6: STATION 6 (Brakspruit)

Small non-perennial river flowing through typical False Upper Karoo vegetation. A limestone ridge across the river is visible in the centre of the photograph. Marginal vegetation consists of scattered tussocks of *Scirpus* sp. (No *A. filiculoides* present since photograph taken in March, after floods.)



PLATE 7: STATION 7 (Suurbergspruit)

Small, non-perennial stream flowing through typical False Upper Karoo vegetation. The marginal vegetation is dominated by dense stands of *Typha* sp. and *Phragmites* sp. with occasional *Acacia karroo* and *Salix* sp.

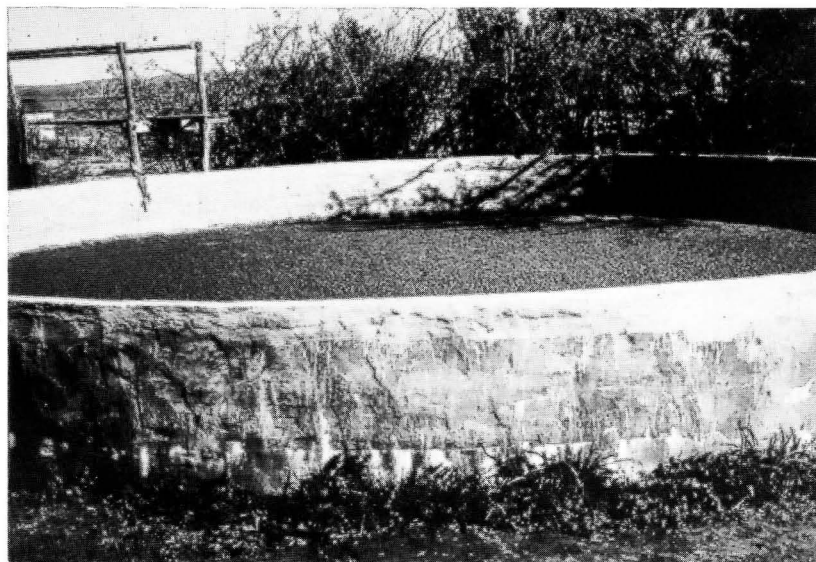


PLATE 8: STATION 8 (Ratelpoort)

Concrete irrigation reservoir, 10 metres in diameter, with a thick mat of *A. filiculoides*. Some *Acacia karroo* growing at edge of reservoir provides partial shade.

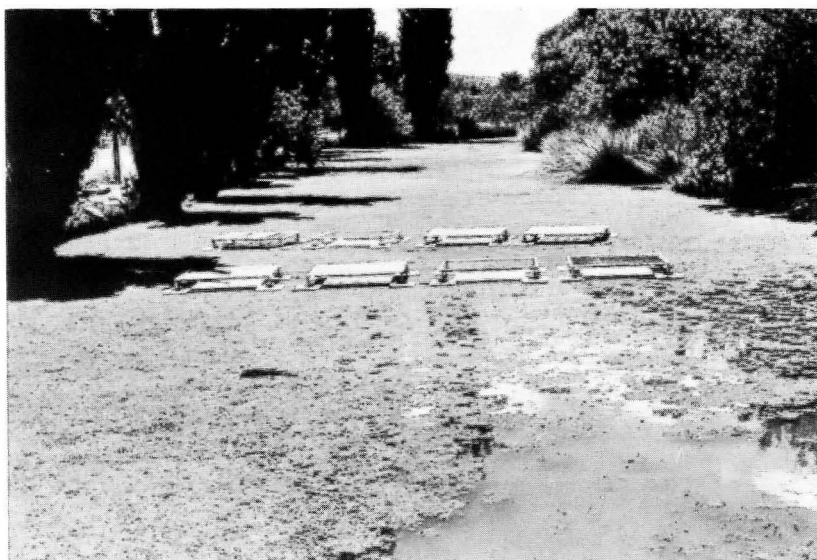


PLATE 9: STATION 9 (Kleintoren Dam)

Farm irrigation dam covered with mat of *A. filiculoides*. Marginal vegetation consists of *Eragrostis lehmanniana* and *Acacia karroo* (right) and *Populus* sp. (left). The floating screens in the centre of the photograph are those used in field growth trials (see close-up in Plate 14).



PLATE 10: STATION 10 (Kleintoren River)

Small perennial river covered with thin mat of *A. filiculoides*. Marginal vegetation consists of *Scirpus* sp. (right foreground), *Eragrostis lehmanniana*, *Cyperus* sp. and *Acacia karroo* (to left and right) and a few large specimens of *Salix* sp. (left).



PLATE 11: STATION 11 (Rietfontein)

Small perennial river flowing through typical False Upper Karoo vegetation. Marginal vegetation dominated by *Phragmites* sp. (left), with some *Scirpus* sp., *Typha* sp. and *Acacia karroo* (right). (No *A.filiculoides* present as photograph taken in March, after floods.)



PLATE 12: STATION 12 (Van Wyksfontein)

Small perennial spring, 6 metres in diameter, in Dolerite formation. Thin mat of *A.filiculoides* growing with *Nasturtium officinale* (Water Cress). Large specimens of *Acacia karroo* around the margin of the spring provide dense shade.

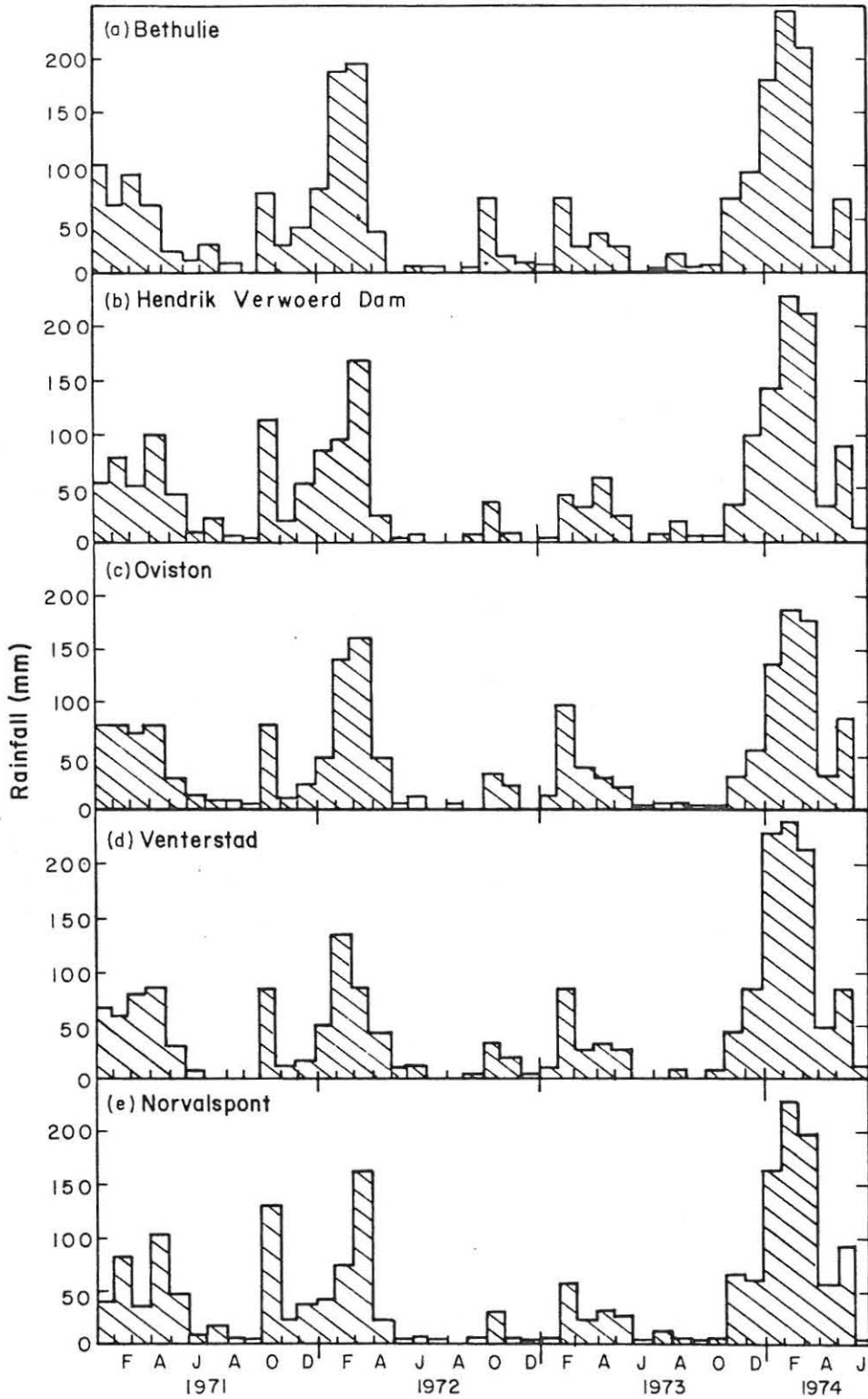


FIG. 5 Histograms of total monthly rainfall recorded at five towns in the study area, from January 1971 to June 1974.

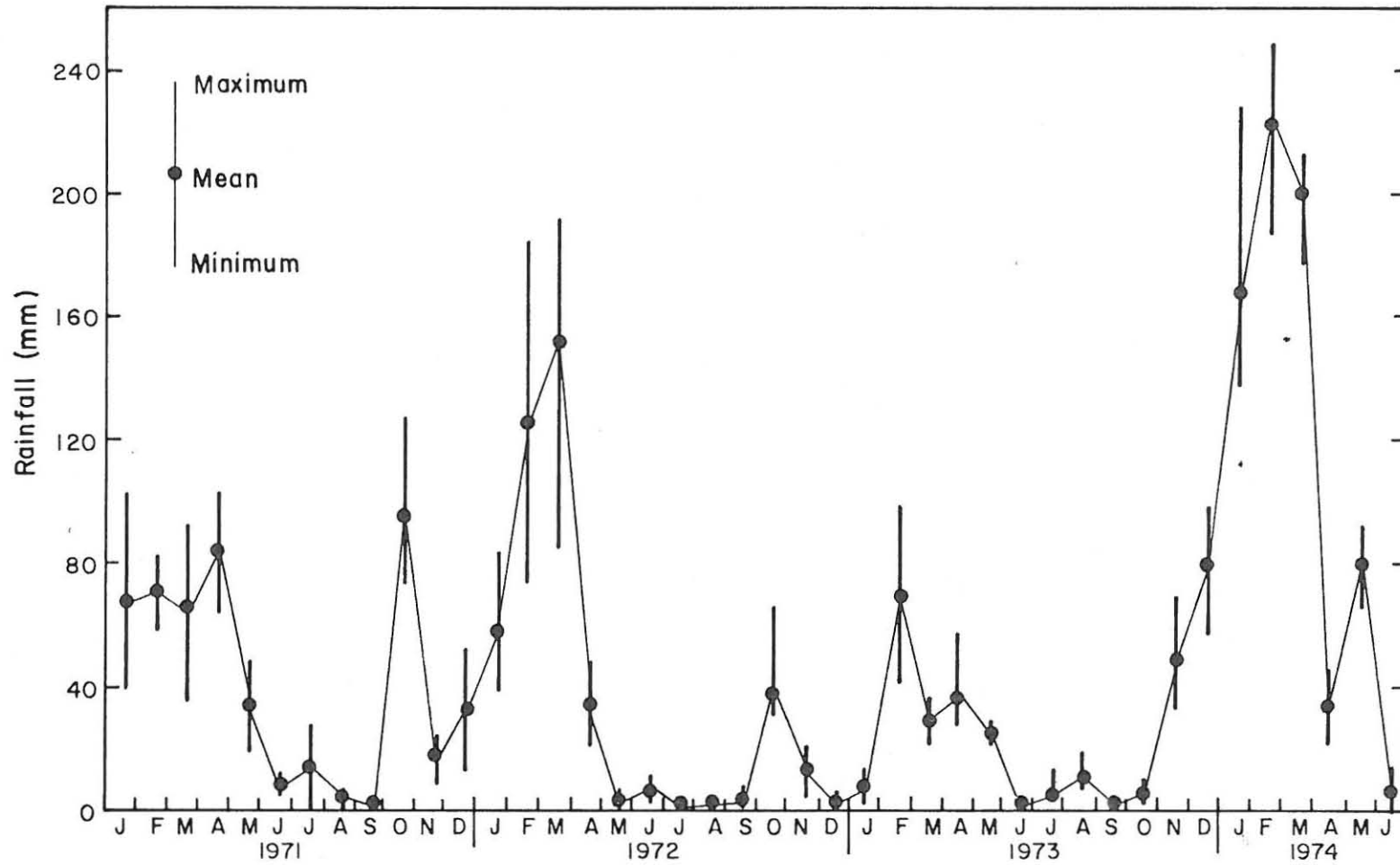


FIG. 6 Variation in maximum, mean and minimum monthly rainfall for five towns (Bethulie, Hendrik Verwoerd Dam, Oviston, Venterstad, Norvalspont) from January 1971 to June 1974.

north-west. Relative humidity (measured by swing psychrometer) is always less than 48 % at midday. Maximum light intensities (measured with a Gossen Trilux Lightmeter at midday) vary from 80 kLux in winter to 115 kLux in summer.

## 2.2 SAMPLING FREQUENCY AND SAMPLE SIZE

The study area was visited at monthly intervals for a period of 40 months between February 1971 and June 1974. All localities known to contain *Azolla filiculoides* were examined on each visit, as well as any nearby *Azolla*-free areas, to check on any changes in the distribution pattern of the plants.

During the preliminary survey in February 1971, a study was carried out to determine the minimum number of plant and water samples that should be collected so as to give the maximum amount of information for the time and effort involved. At each of three localities, ten integrated water samples and 40 plant samples of 0,01 m<sup>2</sup> area were collected. The samples were transported to the laboratory and the water samples analysed for macro- and micronutrients while the biomass and number of plants within each plant sample was determined. From the data obtained, the minimum number of plant samples needed to approximate the 'population' mean value was calculated from the formula of Elliott (1971):

$$N = t_{(0,05)}^2 \cdot \frac{S^2}{D^2 \cdot \bar{x}^2} \quad (1)$$

Where N is the minimum number of samples, t is the Student's t value corresponding to the required confidence level (95 %) and to n-1 degrees of freedom (n being the number of measurements), S<sup>2</sup> is an estimate of the square of the standard deviation (i.e. the variance), D is the allowable error of the mean (in this case 5 %) and  $\bar{x}$  is the arithmetic mean of each data set. For convenience, formula (1) can be reduced to:

$$N = t_{(0,05)}^2 \cdot \frac{S^2}{(D \cdot \bar{x})^2} \quad (2)$$

For the three sets of forty plant samples, N was calculated to be 7,72, 6,35 and 7,86 respectively (see Appendix I), while for the three sets of

ten integrated water samples the value of N was calculated to be 2,65, 2,83 and 2,74 respectively (based on the analyses of the conservative mineral constituents Na, K, Ca and Mg). Therefore, to ensure the minimum sampling error during routine field sampling, ten plant samples and four integrated water samples were collected from each site on each sampling visit.

Formula (2) was also used to calculate the minimum number of replicate samples necessary for laboratory growth trials. Here, N was found to be 6,52 and therefore for all laboratory growth trials, at least eight replicates of each treatment were used.

### 2.3 FIELD SAMPLING, TRANSPORT AND PREPARATION OF SAMPLES FOR CHEMICAL ANALYSIS

At each sampling site, five sets of pH, dissolved oxygen and water temperature profiles were measured. These measurements were taken at randomly selected points within the sampling site and individual readings were taken from 2 cm below the water surface to the bottom at 10 cm intervals. pH values were measured with a Metrohm model E444 portable pH meter with a glass electrode while oxygen and temperature readings were taken with a YSI model 54A portable oxygen meter equipped with a thermistor probe.

The total area of water surface covered by *Azolla* plants within each site was estimated by using a series of 1 m<sup>2</sup> quadrats in line (Ashton, 1974). A square wooden tube, 100 cm<sup>2</sup> in cross-sectional area and 40 cm in length (Ashton, 1974) was then employed to obtain ten replicate samples of plants from the *Azolla* mat at each site for population estimates and chemical analyses. Population estimates were expressed as the mean number of *Azolla* plants per square metre of available water surface. The plants were gently blotted dry with a towel, weighed to 0,1 g on a portable field balance and placed in numbered plastic bags. Air was excluded from the bags by gentle pressure, the bags sealed and then stored on ice in the dark for transport to the laboratory. On return to the laboratory, the plants in each sample were counted and the numbers of plants bearing sporocarps noted. The samples were then weighed, placed in Pyrex glass dishes in a Memmert forced-draught oven and dried at 65 °C for at least

36 hours. The dried plant material was weighed and then ground in a Casella grain mill through a 0,53 mm pore diameter sieve. The resulting powdered material from the ten replicate samples was thoroughly mixed and redried at 65 °C for at least 4 hours before subsamples were taken for chemical analysis (see Section 2.4.1).

Water samples were collected at 25 cm intervals from the surface to the bottom by means of a 2 l polyethylene bottle fixed to a graduated wooden pole. The bottle was lowered to the appropriate depth and by pulling a length of twine attached to a loose-fitting rubber stopper in the neck of the bottle, a water sample was collected. Single 2 l samples from each depth were first filtered through a single layer of cheese cloth to remove coarse organic debris and were then mixed in a 20 l polyethylene bucket to produce an integrated sample. Four such samples were collected at each site. (The prefiltering through cheese cloth did not remove silt or colloidal material which was often present in considerable quantities.) From each integrated sample, a single 2,5 l subsample was collected in an HCl-leached and washed polyethylene bottle. Each sample was immediately stored on ice, in the dark, for transport to the laboratory, a period of storage which was never longer than 28 hours. Although it has been recommended (Golterman and Clymo, 1971) that water samples should be preserved with  $H_2SO_4$  to prevent, or at least minimize changes in the chemical composition of the samples, it was felt that the alternative method (i.e. chilling the samples) would prove to be as effective. This was confirmed by comparing the analysis of  $H_2SO_4$ -preserved samples with that of the routinely chilled samples.

In the laboratory 2,0 l of each sample was filtered through prewashed 0,45 µm Millipore filters, placed in acid-leached and washed polyethylene bottles and frozen to await chemical analysis. The remaining portions of each sample were analysed immediately for conductivity, alkalinity, suspended solids, total nitrogen, total phosphorus and ammonium acetate exchangeable cations (see Section 2.4.2).

## 2.4 CHEMICAL ANALYSIS OF SAMPLES

### 2.4.1 Plant samples

The analytical procedures employed for the analysis of plant samples in this study were those used for the rapid, routine analysis of Pineapple leaf samples by the Rhodes University Pineapple Research Unit (Hasses, 1971). Basically, the procedures are as follows:

#### (a) Metal cations

Duplicate 2,500 g portions of the ground, dried plant material were weighed into clean dry silica dishes and dry-ashed in a muffle furnace at 490 °C for at least 5 hours. The plant ash was then treated with 5 ml of concentrated HCl and evaporated to dryness on a waterbath to dehydrate and precipitate any silica present in the ash. The crystalline residue in each dish was then dissolved in 5 ml of concentrated HNO<sub>3</sub>, diluted with deionized water, filtered through a preleached Whatman No. 541 filter into a 50 ml volumetric flask and made up to volume. This solution was then analysed directly for the cations Mg, Ca, Na, Cu, Zn, Fe and Mn by atomic absorption spectroscopy and K by flame emission spectroscopy. (If cation concentrations were too high, these solutions were diluted with deionized water appropriately.) These analyses were carried out with a Techtron AA 4 atomic absorption spectrophotometer equipped with a D.I. 30 Digital Indicator, an I.M. 5 scale expander facility and a D.I. 32 computerized printout (which permitted the results to be computed directly as concentrations in the original plant sample).

#### (b) Phosphorus

The ash solution obtained from the dry-ashing procedure in (a) above was also used for the spectrophotometric determination of phosphorus in the plant tissue. The method employed was the vanado-molybdo-phosphoric yellow method recommended by Jackson (1958). Here, 3 ml of the ash solution was diluted with 27 ml of a vanado-molybdo-nitric acid reagent containing 25 g (NH<sub>4</sub>)<sub>6</sub>Mo<sub>7</sub>O<sub>24</sub>.420, 1,25 g NH<sub>4</sub>VO<sub>3</sub> and 250 ml of concentrated HNO<sub>3</sub> per litre. The resulting solution was allowed to stand for at least 15 minutes to permit full colour development and the absorbance was read at 445 nm on a Beckman DB spectrophotometer equipped with a sequential sampler. The

advantages of this method are the ease of operation and the fact that the yellow-coloured vanado-molybdo-phosphoric complex is stable for at least 24 hours. However, the method is not accurate for phosphorus concentrations below  $1 \text{ mg l}^{-1}$ .

(c) Micro-Kjeldahl nitrogen analysis

Duplicate 0,100 g portions of the ground, dried plant material were weighed into 30 ml Micro-Kjeldahl flasks. Two grammes of  $\text{K}_2\text{SO}_4$  were added to each flask, followed by 2,5 ml of concentrated  $\text{H}_2\text{SO}_4$  and 0,5 g of  $\text{HgSO}_4$  as catalyst (Steyn, 1957). Digestion of the plant material was accomplished by heating the flasks on an electrical digestion rack in a fume cupboard for 45 minutes. The solutions were cooled and quantitatively transferred to a Buechi steam distillation apparatus followed by the addition of 10 ml of a caustic thiosulphate solution containing 25 g  $\text{Na}_2\text{S}_2\text{O}_3 \cdot 5\text{H}_2\text{O}$  and 400 g NaOH per litre. The mixture was then steam distilled for 12-15 minutes, the distillate (containing the nitrogen in the form of ammonia) collected in a boric acid plus mixed indicator solution and titrated against standard 0,015 M HCl. Duplicate blank samples, containing reagents, but no plant material, were analysed before and after each batch of samples.

The results of the analysis were expressed as percentage nitrogen present in the dried plant material and were calculated from the following formula:

$$\% \text{ N} = \frac{[\text{Vol. HCl}_{(\text{sample})} - \text{Vol. HCl}_{(\text{blank})}] \cdot \text{M HCl} \cdot 14,007 \cdot 100}{\text{sample weight in milligrams}} \quad (3)$$

The advantages of this method are the rapidity of the digest and the fact that unpleasant features of distillation from the mercury digests were avoided by the use of sodium thiosulphate which precipitated the mercury as black mercuric sulphide.

2.4.2 Water samples

The choice of suitable methods for the chemical analysis of water is complicated by the number of manuals that have been written (e.g. Mackereth, 1963; Strickland and Parsons, 1969; American Public Health Association, 1971; Golterman and Clymo, 1971), each book recommending different variations of methods for selected parameters. With the formation of the

International Biological Programme (IBP) and the subsequent publication of the IBP Handbook No. 8 'Methods for Chemical Analysis of Freshwaters' (Golterman and Clymo, 1971), it was decided that the methods advocated in this book would be used since they represented proven methods the results of which could be compared with data obtained from any part of the world.

Careful consideration was given to the geology, soils, climate and land-usage of the study area when deciding which chemical parameters should be measured in the present study. Since the pH of the water is governed by the buffering capacity of the water, the carbon dioxide cycle and the activities of the aquatic biota, its measurement was considered essential. In order to be able to classify the stream and river waters further, as well as to evaluate the nutritional requirements of the *Azolla* plants, the major cations and anions were also determined. These included Ca, Mg, Na, K, Cl and  $\text{SO}_4$ . In addition, the minor cations Cu, Fe, Mn and Zn were also analysed. The analysis of nitrogen and phosphorus was considered essential, since they are the key elements regulating biological activity and therefore both dissolved and particulate nitrogen and phosphorus fractions were determined. Due to the heavy silt load brought down by the rivers after rain, the analysis of ammonium acetate-exchangeable or 'adsorbed' major cations was also carried out.

Except for pH, suspended solids, conductivity, alkalinity, total nitrogen, total phosphorus and ammonium acetate-exchangeable cations, all analyses were performed on filtered samples which had first been thawed.

For methods involving spectrophotometry and atomic absorption spectroscopy, calibration standards were prepared with each series of determinations and periodic checks were carried out between determinations as a precaution against instrumental drift. A generalized scheme showing the analyses carried out on each water sample is shown in Figure 7.

Physical and chemical analyses of water samples were carried out, using the following methods:

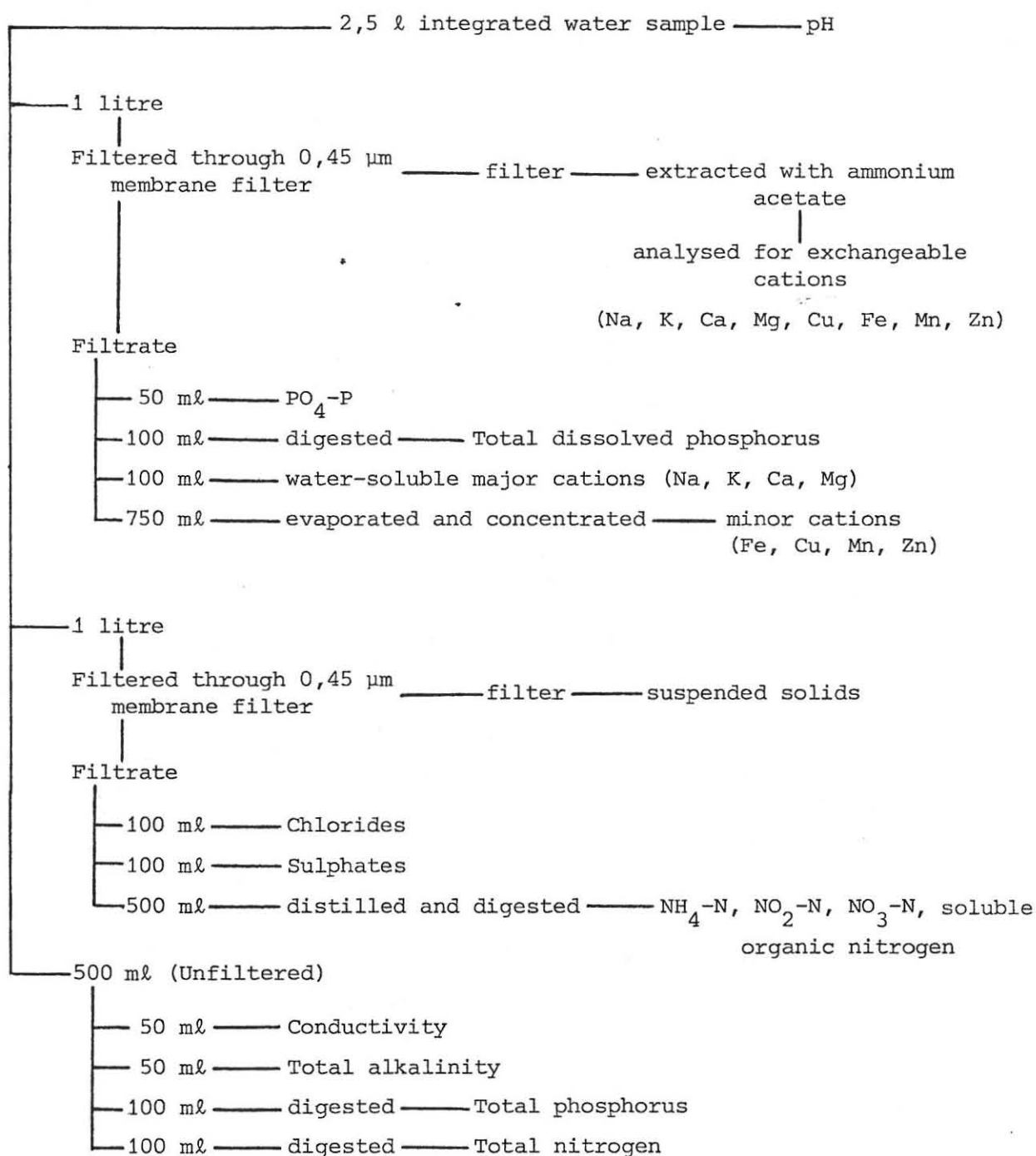


Fig. 7

Schematic representation of the chemical analyses carried out on each water sample

(a) pH

pH was determined in the field as described in Section 2.3, the pH meter being calibrated against standard buffer solutions having pH values of 7,0 and 10,0.

(b) Conductivity

The conductivity of unfiltered water samples was determined using a Beckman conductivity meter with platinum electrodes. Results were expressed as  $\mu\text{mhos cm}^{-1}$  after being corrected to 25 °C with the conversion table listed in Golterman and Clymo (1971).

(c) Total alkalinity

Total alkalinity was determined immediately on arrival at the laboratory by potentiometric titration with standard 0,05N  $\text{H}_2\text{SO}_4$  as titrant and phenolphthalein as indicator. The end-point was determined accurately with a 240 volt Metrohm E 512 pH meter with scale expansion facility. Total alkalinity (T A) was calculated as milli-equivalents per litre from the equation of Golterman and Clymo (1971):

$$T A = \frac{[\text{Volume of titrant (ml)} \times \text{Normality of titrant} \times 1000]}{\text{Volume of sample (ml)}} \quad (4)$$

(d) Suspended solids

Suspended solids were determined on unfiltered samples by the method of Wyckoff (1964). One litre samples were filtered through weighed pre-washed 0,45  $\mu\text{m}$  Millipore membrane filters and the mass of suspended material determined gravimetrically after drying at 105 °C for 12 hours. Results were expressed in milligrams per litre. Where samples had high concentrations of suspended silt and colloidal material, smaller volumes of sample (250 ml) were used.

(e) Sulphate

Sulphate was determined turbidimetrically at 380 nm with an EEL colorimeter using the stabilizers  $\text{BaCl}_2 \cdot 2\text{H}_2\text{O}$  and a 1:2 (v/v) solution of glycerol and ethanol, as recommended by Golterman and Clymo (1971).

(f) Chloride

Chloride was determined by the conductometric titration method of Golterman and Clymo (1971), recommended for low chloride concentrations, using a Beckman conductivity meter with platinum electrodes and standard  $\text{AgNO}_3$  as titrant. The chloride content of the sample was calculated from the formula:

$$\text{Cl}^- \text{ (meq } \ell^{-1}) = \frac{\text{Vol. AgNO}_3 \text{ (m}\ell) \cdot \text{Normality AgNO}_3 \times 1000}{\text{Vol. sample (m}\ell)} \quad (5)$$

$$\text{Cl}^- \text{ (mg } \ell^{-1}) = \text{Cl}^- \text{ meq } \ell^{-1} \times 35,46 \quad (6)$$

(g) Metal cations

In order to obtain as close an approximation as possible of the nutrients 'available' to *Azolla* plants, the concentrations of both the water-soluble and ammonium acetate-exchangeable cations adsorbed onto the exterior of clay particles were determined. The method employed was as follows: One litre of water sample was filtered through a 0,45  $\mu\text{m}$  Millipore membrane filter to obtain a filtrate containing the water-soluble cations and anions. The residue remaining on the filter was then extracted with four 250 ml aliquots of 1,0 N  $\text{CH}_3\text{COONH}_4$  to obtain the ammonium acetate-exchangeable cations (Schellenberger and Simon, 1945). The two solutions containing the water-soluble cations and anions and the ammonium acetate-exchangeable cations respectively were then analysed directly by atomic absorption spectrophotometry using a Techtron AA 4 atomic absorption spectrophotometer calibrated with appropriate standards. In most cases, the water-soluble minor cations (Cu, Fe, Mn and Zn) were in such low concentrations that they could only be determined after concentration of the sample. This was accomplished by evaporating a 750 ml portion of the 'filtrate' to approximately 20 ml and quantitatively transferring this residue to a 50 ml volumetric flask, thus effecting a 15-fold concentration.

(h) Phosphorus

I. Dissolved orthophosphate ( $\text{PO}_4\text{-P}$ ) was determined spectrophotometrically on filtered (0,45  $\mu\text{m}$ ) samples at 690 nm using modification 'A' of the molybdenum-blue method (Golterman and Clymo, 1971).

II. Total dissolved phosphorus was determined as orthophosphate after digestion (hydrolysatation) of the filtered water sample with concentrated  $\text{H}_2\text{SO}_4$ . Subtraction of the orthophosphate (determined in I above) gives the hydrolysable phosphate, which, in unpolluted waters, consists almost entirely of organic phosphorus compounds (Golterman and Clymo, 1971).

III. Total phosphorus was determined by analysing the hydrolysate of unfiltered samples for orthophosphate. Subtraction of the total dissolved phosphorus then gave the concentration of particulate phosphorus (Golterman and Clymo, 1971).

(i) Nitrogen

The dissolved nitrogen forms, ammonia, nitrite plus nitrate and organic nitrogen were determined simultaneously on a single filtered (Whatman GF/C) water sample of 500 ml. This was accomplished by using a Buechi Macro-Kjeldahl distillation apparatus and by carrying out the determinations in three consecutive steps.

I. Dissolved ammonia was determined by titration with standard 0,005 N HCl after steam distillation and collection of the distillate in a boric acid-mixed indicator solution (reagent blanks using ammonia-free distilled water were run between each batch of samples analysed). The quantity of ammonia in the sample was calculated as nitrogen from the formula of Golterman and Clymo (1971):

$$N \text{ (mg } \ell^{-1}) = \frac{(\alpha - \beta) \times \text{Normality acid} \times 1000 \times 14,007}{\text{Vol. sample (ml)}} \quad (7)$$

where:

$\alpha$  = Volume of standard HCl used for sample, and

$\beta$  = Volume of standard HCl used for blank.

II. Nitrate plus nitrite were then reduced to ammonia by the addition of Devarda's alloy under strongly alkaline conditions, followed by steam distillation and titration of the ammonia as described in I. above.

III. Dissolved organic nitrogen was also determined as ammonia by titration (as in I. above), following acid digestion of the residual sample in the Kjeldahl flask.

IV. Particulate nitrogen was determined by analysing the digestion of an unfiltered water sample for ammonia and subtracting from this result the values obtained for dissolved ammonia and dissolved organic nitrogen.

## 2.5 GROWTH ANALYSES

### 2.5.1 Field experiments

In the field, the analysis of *Azolla* growth rates was accomplished with the use of a series of screened floating dishes as shown in Plate 13. Each apparatus consisted of a floating sheet of expanded polystyrene 75 cm x 60 cm x 2,5 cm having twenty circular holes in which 10 cm diameter plastic dishes were placed. Each plastic dish was perforated laterally with ten holes 2,5 mm in diameter which permitted free water movement into and out of the dish whilst preventing the removal of plant material. This float was fastened to and covered by a wooden frame 135 cm x 90 cm fitted with 'Sarlon R' shade cloth, supported by four smaller polystyrene floats at its corners. With the use of different densities of shade cloth, a range of light intensities could be obtained. The height of the wooden frame above the plastic dishes was fixed at 15 cm to prevent shadowing of the plants by the frame and this arrangement permitted virtually unimpeded air movement over the dishes. During the experiments, which were normally of two to three weeks duration, the screens were anchored to a convenient tree or stake on the bank of the stream or farm dam. For the analysis of growth rates, weighed (approximately 1,0 g) samples of washed and blotted *Azolla* plants were placed in each dish and reweighed at 4 day intervals for the duration of the experiment, after which the plants were harvested and dried for chemical analysis.

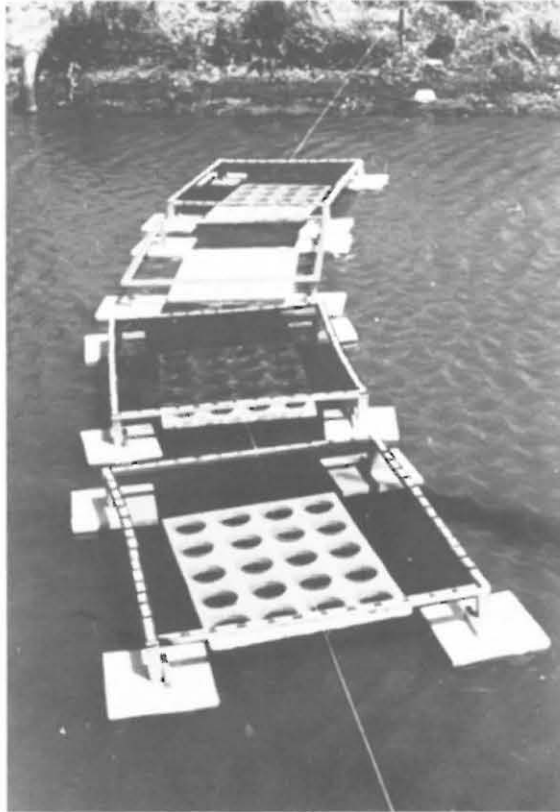


PLATE 13: Apparatus used in field growth trials. The apparatus consists of 4 wooden frames fitted with different densities of Sarlon<sup>R</sup> Shade Cloth. Each frame supported by 4 polystyrene floats at its corners, covers a central polystyrene float supporting 20 perforated plastic dishes containing plants of *A. filiculoides*.

## 2.5.2 Laboratory experiments

### 2.5.2.1 Controlled environment room conditions

All laboratory experiments carried out on the growth and nitrogen fixation activity of *Azolla* plants were conducted in controlled environment rooms (phytotrons) which had internal dimensions of 2,75 m x 2,15 m x 2,30 m and were fitted with adjustable benching. A twelve-point recorder (Honeywell Ltd, London) monitored light intensity, temperature and relative humidity within each of the four rooms used.

Within each room, the light intensity could be varied from 500 Lux to approximately 20 kLux by regulating the numbers of 'cool-white' fluorescent tubes and incandescent strip-lights built into the ceiling of each phytotron. Light intensities greater than this (up to 75 kLux) were obtained by using a bank of twenty-four mercury-vapour photo-flood lamps situated at varying distances from the experimental treatments. An initial serious overheating problem caused by the use of the mercury-vapour lamps was overcome by the use of powerful cooling fans. In each experiment, light intensities were checked daily with a Gossen Trilux light meter (Power Contractors Ltd, Johannesburg).

Three 'Cinamoid' colour filters (G.E.C. Electrical Products, New York), with transmission maxima at 655, 530 and 450 nm were used to obtain light of different spectral ranges. These filters were obtained in sheets measuring 40 x 40 cm and were fitted to the tops of light-proof wooden boxes.

Daylength regimes within each phytotron could be adjusted with a time switch.

Temperatures within each phytotron could be accurately controlled to within 1 °C of the set point, over an effective range of 5 to 45 °C. When changing from one temperature regime to another, temperature stabilization at the new temperature setting was achieved within 45 minutes of the change.

The relative humidity (R.H.) within each phytotron could be accurately controlled from 40 % to 95 % at a wide range of temperature settings. In all experiments, bar those dealing with the effects of desiccation, the relative humidity was kept at 92-95 % to prevent excess evaporation from the culture flasks and dishes.

The atmospheric levels of oxygen, carbon dioxide and nitrogen were kept constant by an automatic intake of a proportion of fresh air by the recirculation fans.

#### 2.5.2.2 Azolla plants

For general experiments, 10 cm diameter plastic dishes were used as culture vessels. Preliminary trials showed that when the roots of *Azolla* plants were suddenly exposed to high light intensities, the *Azolla* plants rapidly shed most of their roots. Therefore, in an effort to prevent this root loss, the exterior surfaces of all culture dishes and flasks were painted with matt black paint up to the water level. This had the additional advantage of preventing excessive periphytic algal growth. The plastic dishes were filled with 450 ml of nutrient solution and had an available surface area of 78,6 cm<sup>2</sup>. In the nutrient deficiency experiments, 1 l capacity Pyrex glass flasks were used. These flasks also contained 450 ml of nutrient solution and, at this level, the available surface area measured 98,6 cm<sup>2</sup>. In the laboratory, plant densities were calculated as the average number of plants per square decimetre of liquid medium surface area and were again based on the means of ten replicates.

Prior to any laboratory growth experiments, the *Azolla* plants were grown in large asbestos trays and acclimatized under the required experimental conditions for three weeks. After acclimatization, the plants were surface-sterilized by treatment for 15 minutes with a 1 % (w/v) solution of NaOCl containing 0,1 % (v/v) teepol as a wetting agent, followed by washing in large volumes of sterile distilled water. During both the acclimatization and experimental periods, culture solutions were replaced daily by siphoning to avoid disturbance of the plants and to ensure constant nutrient supply. Where necessary, the pH of the culture solution was adjusted with 1 N HCl or 1 N NaOH.

For the analysis of mean relative growth rates ( $\overline{\text{RGR}}$ ), weighed samples (approximately 1,0 g) of surface-sterilized plants were placed in nutrient solution and reweighed seven days later. The  $\overline{\text{RGR}}$  was calculated on a dry weight basis (after correction for water content of the plant material) and was based on the formula of Radford (1967):

$$\overline{\text{RGR}} = \frac{\ln \text{ Final Weight} - \ln \text{ Initial Weight}}{\text{Time Interval (days)}} \quad (8)$$

$$= \frac{\ln (\text{Final Weight}/\text{Initial Weight})}{\text{Time Interval (days)}} \quad (9)$$

From the mean relative growth rate, the doubling time (in days) was calculated using the formula of Mitchell (1974):

$$\text{Doubling Time} = \frac{\ln 2}{\overline{\text{RGR}}} \quad (10)$$

In experiments to determine the effects of wave action, *Azolla* plants were grown in 300 ml Erlenmeyer flasks containing 100 ml of nutrient solution and were shaken at different rates on a Bühler rotary shaker with an orbit radius of 3 cm.

#### 2.5.2.3 Anabaena azollae

The growth rate of cultured *Anabaena azollae* was determined, on a dry weight basis (Kulasooriya, Lang and Fay, 1972), using the formula for mean relative growth rate (9) above. Due to the relatively slow growth rate of the alga in culture, large initial inocula (approximately 1,0 g wet weight) were used in growth experiments. As in laboratory experiments with *Azolla* plants, a minimum of ten replicates of each treatment was used where possible. The weighed algal inocula were placed in 500 ml Erlenmeyer flasks containing 200 ml of sterile nutrient solution. The flasks were shaken at 20 revolutions per minute on a Bühler rotary shaker with an orbit radius of 3,0 cm and reweighed at seven day intervals.

#### 2.5.3 Statistical analyses

Data manipulation and statistical computations, including means, standard errors, analysis of variance, F test probability and regression analysis

were performed using the SPSS computer software package (Nie, Hall, Jenkins, Steinbrenner and Bent, 1975) on the CSIR's CDC Cyber 174 computer.

## CHAPTER 3

### TAXONOMY AND DISTRIBUTION

#### 3.1 INTRODUCTION

The first classifications of *Azolla* species were based on vegetative characteristics, in particular leaf form and size (Svenson, 1944). This led to considerable confusion since the morphology of *Azolla* plants is often modified by interacting environmental influences (Pieterse, De Lange and Van Vliet, 1977). Subsequent investigations demonstrated that both extant and fossil species of *Azolla* could be separated much more easily on the basis of sporocarp structure (Svenson, 1944; Sweet and Hills, 1976).

The six extant species of *Azolla* are widely distributed through most of the temperate and tropical regions of the world and at least two of these species have been introduced into areas where they are not indigenous (Lumpkin and Plucknett, 1980).

This investigation was designed to identify positively the *Azolla* species occurring in the catchment area of the Hendrik Verwoerd Dam and define the distribution of all *Azolla* species in southern Africa.

#### 3.2 LITERATURE REVIEW

##### 3.2.1 Fossil record

The fossil record of the genus *Azolla*, dating back to the late Cretaceous of North America (Hall, 1969) is based mainly on its highly distinctive reproductive structures, the megaspore apparatus and microsporocarpic massulae, which are considered the most complex of any heterosporous plants (Hall, 1974). To date the fossils of two extant and more than forty extinct species of *Azolla* have been described, many of which have been established on very limited information (Fowler, 1975a). Taxonomic diversity within the genus, coupled with the short stratigraphic ranges of individual species, renders *Azolla* a potentially valuable stratigraphic indicator (Follieri, 1970-71; Fowler, 1975a).

Only two of the extant species of *Azolla*, *A. filiculoides* Lam. and *A. pinnata* R. Br., have fossil records. These fossils were found in Pleistocene deposits in Europe (Hills and Gopal, 1967), Australia (Schofield and Colinvaux, 1969) and more recently in Japan (Seto and Nasu, 1975). Although not indigenous to Europe in recent times (Sculthorpe, 1967), *A. filiculoides* has been found as a fossil in the Second (Great) Interglacial deposits in Britain (West, 1953; Charlesworth, 1957), Germany (Rochow, 1955), Italy (Follieri, 1970-71), Poland (Moore, 1969), Russia (Kornilova, 1953) and Scandinavia and Holland (Charlesworth, 1957). Thus *A. filiculoides* "..... appears to have extended from Europe through northern Asia to North America during the Second Interglacial period ....." (Moore, 1969), but was exterminated in Europe during the Third Glaciation since no evidence of its occurrence in the Third (Last) Interglacial has been found (Charlesworth, 1957).

In recent years, several contributions have appeared on the evolution of the genus *Azolla* and related fossil genera in the Salviniaceae. Most taxonomic accounts of fossil *Azolla* have attempted to demonstrate a direct phyletic lineage based on the number and arrangement of floats on the megaspore apparatus, and, in more recent studies using the electron microscope, features of the stratification and ornamentation of the sporoderm and perine (Jain, 1971; Hall, 1974; Fowler, 1975a; Sweet and Hills, 1976; Martin, 1976a).

In summary, the most striking feature about the evolution of the Salviniaceae is its sudden appearance and differentiation in the late Cretaceous followed by its almost equally sudden decline. The genera *Glomerisporites*, *Azollopsis*, *Azolla* and *Parazolla* all first occur in the Senonian but only *Azolla* survived beyond the end of the Cretaceous. The related genus *Salvinia*, which appeared in the Maastrichian, also survived to the present.

### 3.2.2 Taxonomy and distribution of the genus *Azolla*

The genus *Azolla*, established by Lamarck in 1783 (Svenson, 1944), is a coherent group (Demalsy, 1953), clearly separated from *Salvinia* Adans., the other genus with which it is usually associated to form the Salviniaceae

(Sadebeck, 1902; Sim, 1915; Black, 1948; Bailey, 1949; Lawrence, 1951; Benson, 1957). More recent authorities (Melchoir and Werdermann, 1954; Reed, 1954; Eichler, 1965; Sculthorpe, 1967; Schelpe, 1970; Konar and Kapoor, 1974; Martin, 1976b) have assigned *Azolla* to a monogeneric family, the Azollaceae, separate from the genus *Salvinia*. Various origins for the name *Azolla* have been suggested (Bailey, 1902; Black, 1948; Bailey, 1949). However, it is generally considered that the generic name, *Azolla*, is a conjugation of two Greek words, *Azō* (to dry) and *ollyō* (to kill), inferring that the fern is killed by drought (Moore, 1969; Lumpkin and Plucknett, 1980).

Differentiation at the species level is less clear-cut. Sadebeck (1902) based his treatment of the genus on Strasburger's monograph of 1873, in which two sections (subgenera) were recognised. These subgenera, *Azolla* and *Rhizosperma*, were based on the number of floats (Schwimmkörper) on the megaspore (Svenson, 1944; West, 1953). Sadebeck (1902) cited the author of *Rhizosperma* as Meyen, but Airey Shaw (1966) cited *Rhizosperma* Meyen as a generic name (a synonym for *Azolla*). Meyen's work has, however, not been seen by the author. Sweet and Hills (1976) state that in 1836 Meyen grouped the extant species of *Azolla* (*sensu lato*) into two separate genera *Euazolla* and *Rhizosperma*, the genus *Rhizosperma* being reduced by Mettenius in 1847 to sectional rank within the genus *Azolla*, after which Strasburger, in 1873, referred to *Rhizosperma* as a subgenus. Subsequently, some authors (Demalsy, 1958; Kempf, 1969; Moore, 1969) have used the rank of subgenus to define infrageneric taxa within *Azolla*, whereas others (Rao, 1936; West, 1953; Dorofeev, 1959; Hall, 1968; Hills and Gopal, 1967; Jain, 1971; Bertelsen, 1972; Fowler, 1975b; Martin, 1976a; Lumpkin and Plucknett, 1980) have persisted in the use of the rank of section for infrageneric taxa within the genus *Azolla*.

In an attempt to stabilize the usage of and give meaning to the ranks of subgenus and section within *Azolla* and related genera (both fossil and extant), Sweet and Hills (1976) proposed a dual hierarchy. Their suggestion is that subgenera be defined by the type of glochidia (or homologous structures) that are borne by the massula and that sections be limited to groups of species having the same gross morphology of the megaspore complex and (by implication) the same basic glochidial form.

Therefore, within extant species of the genus *Azolla*, two subgenera are presently recognized, *Azolla* and *Rhizosperma* (Meyen) Strasburger, to account for forms having fluked glochidia and trichome-like glochidia respectively.

Hills and Gopal (1967), dealing with fossil forms of *Azolla*, state that there are six extant and twenty-five fossil species. Svenson (1944) has given a critical account of the four New World species (subgenus *Azolla* (Meyen) Strasburger), using features of the reproductive organs as criteria. According to Svenson (1944), identification of sterile material is often extremely difficult.

The following treatment of extant forms of the genus is based primarily on the works of Sadebeck (1902), Svenson (1944) and Sweet and Hills (1976):

Subgenus *Azolla* (Meyen) Strasburger [Section *Euazolla* Meyen (*Sensu* Sadebeck)] (Three 'floats' on the megaspore; rigid, barbed glochidia on the microsporangial massulae.)

*Azolla filiculoides*: Glochidia not septate, or rarely with 1 or 2 septae at apex; plants elongate (frequently 20-50 mm long), with closely appressed, imbricate, papillose, oblong to ovate leaves (1-2 mm long); microsporangia 35-100 in an indusium; massulae 4-6; megasporangia with raised, irregularly hexagonal markings.

*Azolla caroliniana*: Glochidia not septate; plants small (5-15 mm diameter), dichotomously branched, the nearly orbicular, divaricate leaves small (5 mm long), nearly smooth, not closely imbricate; microsporangia 8-40 in an indusium.

*Azolla mexicana*: Glochidia multi-septate; plants dichotomously branched (10-15 mm diameter), with upper leaf lobes 0,7 mm long, the lower lobes much larger; microsporangia usually with 4 massulae; megaspore pitted.

*Azolla microphylla*: Glochidia multi-septate; plants small (10-20 mm long), pinnately branched, with nearly orbicular leaves 1 mm long; megaspore smooth.

*Azolla filiculoides* Lam., the type species of the genus, is widely distributed, having been introduced to a number of areas in which it is not indigenous. Prior to its dispersal by man, the species was endemic to southern South America and western North America (Lumpkin and Plucknett, 1980). It has since been reported throughout the Americas from southern South America to Alaska (Svenson, 1944), Hawaii (Fosberg, 1942), China (Reed, 1954), Japan (Seto and Nasu, 1975), Australia (Bailey, 1902; Black, 1948; Learmonth, 1953), New Zealand (Bailey, 1902), Belgium (Lawalree, 1964), Holland (Sculthorpe, 1967), France (Chevalier, 1926), England (Williams, 1943, 1944; Smith, 1971), Ireland (Brunker, 1949), Alsace (Jaeger, 1951-52), Portugal (Reed, 1962), Romania (Lawalree, 1964), Germany (Schloemer, 1953), Bulgaria and Czechoslovakia (Sourek, 1958) and South Africa (Twyman and Ashton, 1972). *Azolla rubra* R. Br. is listed by Eichler (1965) as a variety, *Azolla filiculoides* var. *rubra* (R. Br.) Strasb. *Azolla japonica* Franch et Sav., occurring in Japan, is similar to *Azolla filiculoides* Lam. (Moore, 1969) and is also considered to be a variety of *A. filiculoides* (Lumpkin and Plucknett, 1980).

*Azolla caroliniana* Willd. is indigenous to the eastern United States (Bailey, 1902; Svenson, 1944; McCoy, 1950; Reed, 1951; Cohn and Renlund, 1953; Shaver, 1954; Duncan, 1960), the West Indies (Svenson, 1944; Bailey, 1949) and Mexico (Rzedowski, 1957). This species has been introduced into eastern Spain (Bolos and Masclans, 1957), Portugal (Wild, 1961), France (Massol, 1950), Hong Kong (Herklots, 1940), Belgium, Bulgaria, Czechoslovakia, Holland, Hungary, Romania (Lawalree, 1964) and Denmark (Olsen, 1972).

*Azolla mexicana* Presl. occurs from northern South America to British Columbia and eastwards to Illinois (Svenson, 1944; Gunning and Lewis, 1957; Fowler, 1975a).

*Azolla microphylla* Kaulf. occurs in western and northern South America and the West Indies (Svenson, 1944) and the Galapagos Islands (Schofield and Colinvaux, 1969).

Subgenus *Rhizosperma* (Meyen) Strasburger [Section *Rhizosperma* Meyen (*Sensu* Sadebeck)]. Nine 'floats' on the megaspore; glochidia on the microsporangial massulae thread-like (trichome-like) or absent.

*Azolla pinnata*: Glochidia thread-like, present only on the inner surface of massulae; sporocarps in pairs; stems pinnate or bipinnate, plants appearing deltoid in outline, 10-20 mm in length; leaves papillose (each papilla 2-celled), ovate, obtuse, loosely imbricate, 0,8-1,2 mm long; numerous root hairs on each root.

*Azolla nilotica*: Glochidia absent from massulae; sporocarps in tetrads; stems elongated (60-150 mm long); branches often arising in tufts from main axis; leaves nearly smooth, obtuse, not closely imbricate, 3-5 mm long.

*Azolla pinnata* R. Br. is widespread throughout the eastern hemisphere. It has been reported from tropical Africa (Bailey, 1902; Sadebeck, 1902), southern Africa and Madagascar (Sadebeck, 1902; Oosthuizen and Walters, 1961; Wild, 1961; Schelpe, 1970, 1971), West Africa (Sweet and Hills, 1971), Australia (Bailey, 1902; Black, 1948), New Caledonia (Sadebeck, 1902, Indonesia (Sadebeck, 1902; Burkill, 1935; Saubert, 1949), Ceylon (Sadebeck, 1902), India (Rao, 1936; Srivastava and Tandon, 1951; Loyal, 1958; Singh, 1961; Konar and Kapoor, 1972), Indo-China (Vietnam) (Chevalier, 1926), China and Taiwan (Shen, 1960) and Japan (Sadebeck, 1902; Svenson, 1944). Sculthorpe (1967) reports that *Azolla pinnata* has been introduced into southern Europe. Shen (1960, 1961) considers that *Azolla imbricata* (Roxb.) Nakai and *Azolla africana* Desv. are synonyms for *Azolla pinnata* R. Br. After careful examination of a series of collections, Sweet and Hills (1971) agreed, but recognized two varieties on the basis of vegetative morphology. These are *Azolla pinnata* var. *pinnata* occurring in Africa, Madagascar, Australia and New Guinea, and *Azolla pinnata* var. *imbricata* (Roxb.) Bonap., occurring in India, Nepal, Burma, Southeast Asia, China, Phillipines and Japan.

*Azolla nilotica* Decne. is reported as a large species occurring on the Nile (Sadebeck, 1902; Chevalier, 1926), South West Africa (Wild, 1961), Malawi (Reed, 1965), Tanzania (Demalsy, 1953), Mozambique (Wild, 1961; Schelpe, 1971), Uganda (Reed, 1965) and Zambia (Kornas, 1975).

### 3.2.3. The taxonomy of *Anabaena azollae* Strasburger

According to Moore (1969), the endophytic, heterocystous blue-green alga *Anabaena azollae* Strasb. is apparently the only species present in the leaf cavities of all extant species of *Azolla*. In the earliest description of the alga [Strasburger's monograph of 1873, cited in Moore (1969)], Strasburger referred to the alga as *Nostoc* 'strings' (*Nostoc*-Schnüre). Sadebeck (1902) also referred to the alga as the '*Nostoc* parasite'. Strasburger's original description of *Anabaena azollae* in 'Das Botanische Praktikum' [ersten Auflage, Blad 382, cited in De-Toni (1907)], was not available. In a later edition of the above work, Strasburger (1913) included a description sufficiently detailed to comply with requirements specified by the International Code of Botanical Nomenclature (Stafleu, 1972). However, Fjordingstad (1976) claimed that, on morphological grounds, the alga is actually an ecoform of *Anabaena variabilis* and should, therefore, be called *A. variabilis* status *azollae*. This suggestion has, however, not gained wide acceptance.

Taxonomists place *Anabaena azollae* within the phylum Cyanophyta, order Nostocales, family Nostocaceae. The species has sinuous trichomes composed of barrel-shaped cells, which are either sheathless or have a poorly-developed sheath (Geitler, 1925; Shen, 1960). Within each trichome, three types of cells can be found - vegetative cells, heterocysts and thick-walled resting spores or akinetes. In many specimens, akinetes are only found in algal samples from the older senescent *Azolla* leaves. The dimensions of the three cell types are very variable, even within the same sample. There appears to be some slight differences in the dimensions of *A. azollae* cells taken from different species of *Azolla*, though this is not sufficient to separate them into different species or forms (Lumpkin and Plucknett, 1980).

## 3.3 METHODS

### 3.3.1 Collection of samples for identification

At all sites where *Azolla* plants were found, samples were collected for identification. Since the identification of sterile *Azolla* material is at best inconclusive (Svenson, 1944), all localities were sampled during

the summer months November to January when fertile material was available. Samples were preserved with form-acetic-alcohol (FAA) and examined with a Wild M5 stereoscopic microscope on return to the laboratory. Temporary slide mounts were made of squashed microsporocarps to facilitate examination. Where necessary, salient features were photographed with a Zeiss research photomicroscope. Identification of *Azolla* material to species level was based on the works of Demalsy (1953), Shen (1960) and Svenson (1944). In addition, preserved samples of fertile *Azolla* material were dispatched to Dr Rolla M. Tryon, Grey Herbarium at Harvard University, for confirmation of identification.

### 3.3.2 Plant distribution

The data representing distribution records for three species of *Azolla* in southern Africa were compiled from herbarium records, published reports and articles on the plants concerned, information from reliable observers and collections by the author. B.R. Davies, A. Jacot Guillarmod and D.J. Steyn have provided useful data in this regard.

## 3.4 RESULTS

### 3.4.1 Identification of *Azolla* material from the study area

Discussions with farmers in the Colesberg District, South Africa, indicate that *Azolla* plants have occurred in the area for at least the last thirty years. However, the specific identification of these plants was not established until 1972.

Wild (1961) stated that the species concerned was *A. pinnata* R. Br. var. *africana* (Desv.) Bak. This identification, apparently based on the examination of sterile material, is very unlikely, since even in the vegetative state, the plants do not show the typical pinnate branching of *A. pinnata*. Schelpe (1971) identified the species as *A. caroliniana* Willd., again on the basis of sterile material.

After examination of mega- and microsporocarpic material from fifty-seven sites in the Colesberg, Venterstad and Bethulie districts, Twyman and Ashton (1972) have shown that the material belongs, in fact, to *Azolla*

*filiculoides* Lam. This identification is supported by Dr Rolla M. Tryon of the Grey Herbarium at Harvard University (personal communication).

Sporocarps occur in pairs (Plate 14a), usually one megasporocarp and one microsporocarp. Each megasporocarp contains a single megasporangium with one megaspore (Plate 14b). Each megaspore possesses three 'floats' (Plate 14c), which places this material in the subgenus *Azolla* (*sensu* Sweet and Hills, 1976). This rules out the possibility of the material belonging to *A. pinnata*, which has nine 'floats' per megaspore (Sadebeck, 1902; Moore, 1969) and belongs to the subgenus *Rhizosperma* (*sensu* Sweet and Hills, 1976).

Each microsporocarp contains numerous stalked microsporangia (Plate 14d). Examination of the microsporocarpic material showed the presence of numerous non-septate or rarely uni-septate, barbed glochidia on each massula within the microsporangia (Plate 14e, f). Barbed glochidia are a characteristic of the subgenus *Azolla* - the subgenus *Rhizosperma* being characterized by weak, thread-like, unbarbed, trichome-like glochidia on the microsporangial massulae.

The leaves of the material from Colesberg, Venterstad and Bethulie are closely appressed, imbricate, papillose and oblong in shape, being 1-1½ mm in length (Frontispiece). These features are characteristic of *A. filiculoides*, the leaves of *A. caroliniana* being orbicular, divaricate, nearly smooth and approximately 0,5 mm in length (Svenson, 1944). Further characteristics of *A. filiculoides*, shown by the material examined, are the generally elongated shape of the plants (often being two to four centimetres in length), the large number of microsporangia (average 73) within each indusium and the irregular, raised hexagonal markings on the megaspore (Plate 14b).

#### 3.4.2 The distribution of *Azolla* species in southern Africa

The distribution ranges of the three species of *Azolla* that occur in southern Africa are shown in Figure 8. Each symbol marked on the map indicates the presence of *Azolla* within a quarter-degree grid square.

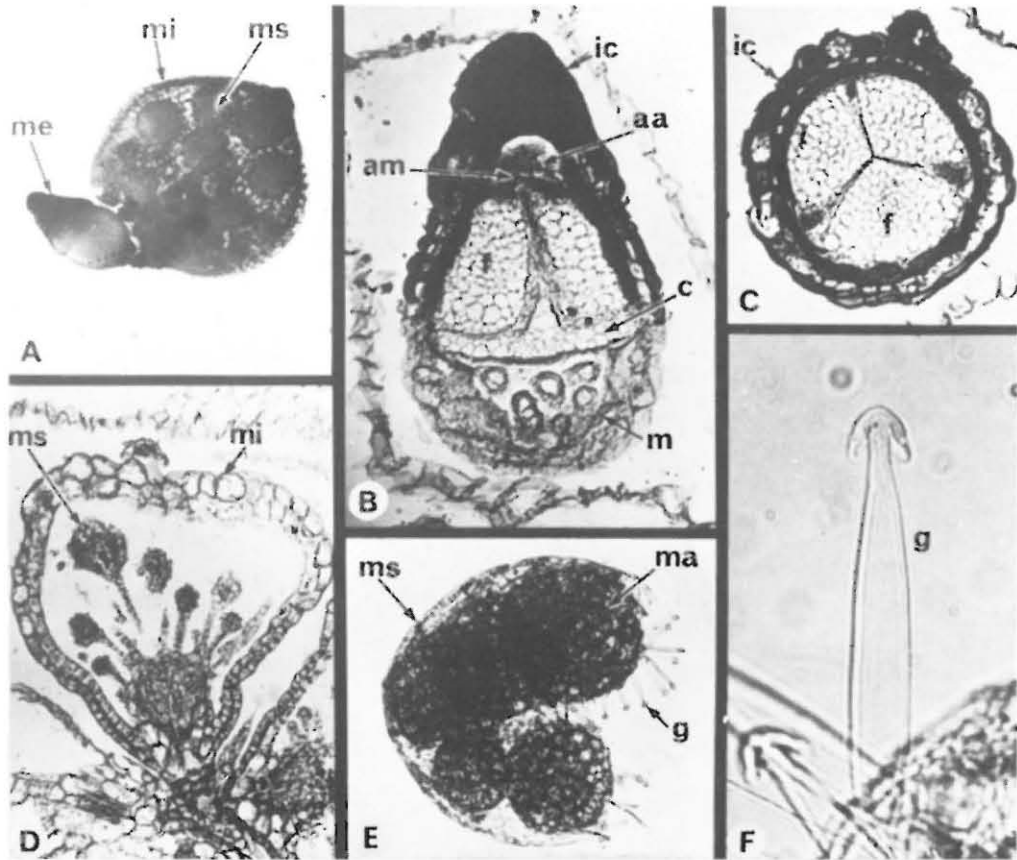
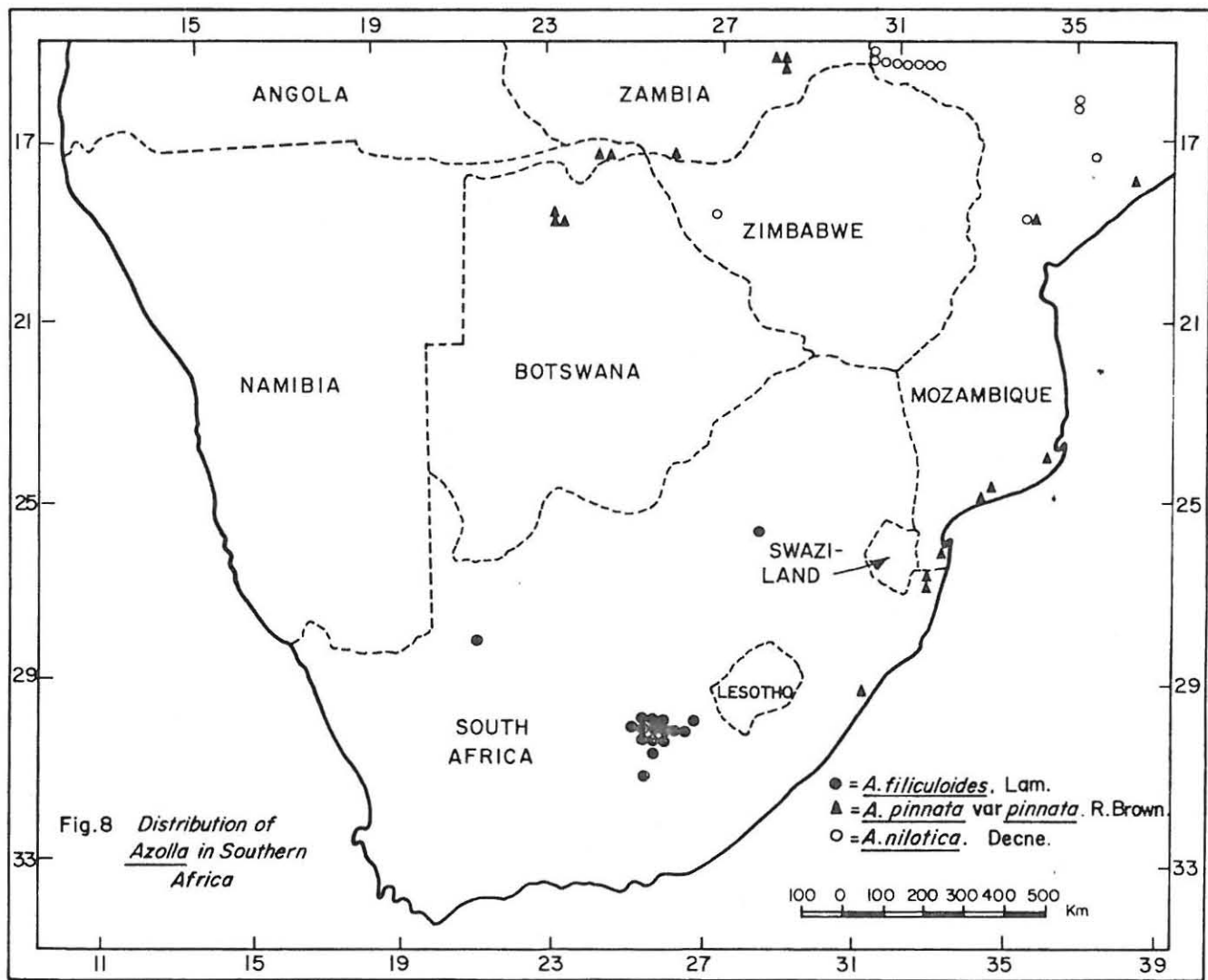


PLATE 14: Structure and arrangement of the reproductive organs in *A. filiculoides* from the Hendrik Verwoerd Dam.

A (X13) Megasporocarp (me) and microsporocarp (mi) containing microsporangia (ms). B (X60) Tangential LS of megasporocarp showing megaspore (m) with apical membrane (am) holding floats (f) onto collar (c). Also shown is the space under the indusium cap (ic) where akinetes of *Anabaena azollae* (aa) are found. (After Ashton & Walmsley, 1976.) C (X70) TS of megaspore "swimming apparatus" showing three float (f) arrangement bounded by indusium cap (ic). D (X30) LS of immature microsporocarp (mi) containing stalked microsporangia (ms). E (X100) Ruptured microsporangium (ms) showing five massulae (ma) bearing fluked glochidia (g). F (X1000) Single, aseptate fluked glochidium (g), typical of *A. filiculoides* Lam.



The distribution of *A. filiculoides* Lam. is very restricted, though within its range it may be locally common. At present, the greatest concentration of the plant is located in the vicinity of the Hendrik Verwoerd Dam, in the Orange Free State and North Eastern Cape Provinces of South Africa. An outlier locality some 450 km downstream of the impoundment, at Uppington, is probably due to the spread of water-borne spores. A further outlier locality in the Florence Bloom Bird Sanctuary, central Johannesburg, Transvaal Province, is the result of human introduction of a so-called 'duck weed' (N. Bloom, personal communication).

*Azolla pinnata* var. *pinnata* R. Br., the only species of *Azolla* indigenous to South Africa, has a very scattered distribution. The plant is found in the Chobe, Linyanti and Kafue river systems of the upper Zambezi River and a few sheltered marshy depressions along the south-eastern coastal plain of Mozambique and South Africa.

*Azolla nilotica* Decne., the largest species of the genus, occurs mainly in sheltered backwaters of the lower Zambezi River and small isolated marshes on the Mozambique coastal plain. The greatest concentration of the plant is to be found in sheltered embayments of the Cabora Bassa impoundment on the Zambezi River in Mozambique (P.J. Ashton, unpublished data).

### 3.5 DISCUSSION

Despite disagreement between taxonomists on the validity of the ranks of subgenus and section within the genus *Azolla*, the use of section is now widely accepted (Lumpkin and Plucknett, 1980). Differentiation at the species level is based on the structure of the reproductive organs since the identification of sterile material is often extremely difficult (Svenson, 1944).

Mega- and microsporocarpic *Azolla* material from the Hendrik Verwoerd Dam catchment area revealed some variability in both the numbers of microsporangia per microsporocarp (24 to 117) and numbers of massulae per microsporangium (6 to 10). However, these values fall within the ranges given by Svenson (1944) for *A. filiculoides*. The presence of non-septate

or (rarely) uni-septate, barbed glochidia on each massula and the irregular, raised hexagonal markings on the megaspore wall, characteristics of *A. filiculoides*, provide additional evidence. Thus, all *Azolla* material collected in the catchment area of the Hendrik Verwoerd Dam has been positively identified as *Azolla filiculoides* Lam. Earlier identifications of sterile *Azolla* material from this area as *A. pinnata* by Wild (1961) and *A. caroliniana* Schelpe (1971) are therefore incorrect.

All southern African *Azolla* material examined (*A. filiculoides*, *A. pinnata* and *A. nilotica*), contained colonies of the endosymbiotic blue-green alga *Anabaena azollae* within cavities in the upper leaf lobes. However, *A. nilotica* plants contained far fewer algal colonies per leaf cavity than did plants of *A. filiculoides* and *A. pinnata*. In all cases, the *Anabaena azollae* material from the three *Azolla* species appeared to be morphologically identical.

The three species of *Azolla* in southern Africa have very restricted distributions, confined to quiet pools or backwaters where they are protected from wind and wave action. The limited distribution of the introduced species *A. filiculoides* within the catchment area of the Hendrik Verwoerd Dam reflects the scarcity of suitable habitat within this area. However, the two outlier localities at Upington and Johannesburg (Figure 8) demonstrate the ease with which the plant may be transported from one locality to another.

## CHAPTER 4

### MORPHOLOGY, ANATOMY AND LIFE HISTORY

#### 4.1 INTRODUCTION

*Azolla* is a genus of small aquatic ferns with a world-wide distribution. Within the genus, extreme reduction in size of organs accompanied by increased adaptation to a free-floating habit, has obscured morphological affinities with other pteridophytes (Sculthorpe, 1967). Indeed, Smith (1938) indicated that the vegetative structure of *Azolla* fronds was reminiscent of certain liverworts. *Azolla* plants superficially resemble members of the Marsiliaceae in being heterosporous and bearing sporocarps. However, *Azolla* differs from the Marsiliaceae in so many morphological features, that they cannot be considered to have any close affinities (Smith, 1938).

Both the vegetative and sexual reproductive cycles in *Azolla* are adapted for existence in an aquatic environment. While much work has been carried out on factors affecting the vegetative growth of the fern (e.g. Peters *et al.*, 1980), nothing has been published about environmental regulation of the equally important processes of sexual reproduction in *Azolla*.

The presence of the heterocystous blue-green alga *Anabaena azollae* within cavities in the dorsal leaf lobes of *Azolla* plants is the only known association between a pteridophyte and a cyanophyte (Stewart, 1978). Earlier studies have shown that this unique association is maintained throughout the life-cycle of the fern. However, the means by which this fern-alga association is maintained during both the vegetative and sexual reproductive cycles is uncertain.

The aims of this investigation were threefold:

- (a) to determine whether the anatomy and life-cycle of *A. filiculoides* plants from the study area differed from *A. filiculoides* plants elsewhere;

- (b) to define the factors responsible for regulating sporocarp production and germination; and
- (c) to investigate the processes of cell development and differentiation in *Anabaena azollae* growing within *Azolla* plants, and the means by which the fern-alga association is maintained.

#### 4.2 LITERATURE REVIEW

##### 4.2.1 Morphology and cytology

When viewed from above, *Azolla* plants are usually triangular or polygonal in shape and float on the water surface individually. When present in large numbers, the plants form dense, multi-layered mats, which give the appearance of a dark green or reddish carpet. The reddish-brown colour, present in all extant species of *Azolla* except *A. nilotica*, is due to the anthocyanin pigments luteolinidin and apigenidin (Pieterse, De Lange and Van Vliet, 1977). The diameter of *Azolla* plants ranges from 10-25 mm for small species such as *A. caroliniana* and *A. pinnata* to 150 mm or more for *A. nilotica* (Demalsy, 1953). The latter species has leafy fronds spaced on a long, trailing, leafless stem (Sadebeck, 1902).

The *Azolla* sporophyte consists of a branched, floating rhizome with small, alternate, overlapping, papillose leaves. Simple roots, which hang down into the water, occur at some nodes. The structural relationships between *Azolla* and other ferns are difficult to discern because of the changes resulting from adaptation to a floating habit (Smith, 1938). Each leaf is divided into a thick, green, dorsal lobe and a thin, colourless, ventral lobe of slightly larger size. The papillose dorsal lobes are chlorophyllous except in the colourless margin. Some authors (e.g. Sadebeck, 1902; Huneke, 1933; Smith, 1938; Bortels, 1940; Saubert, 1949) state that the upper lobe floats on the water surface, the lower lobe being submerged. However, Rao (1936), Svenson (1944), Schaedé (1947), Moore (1969) and most later workers have indicated that the lower lobe is in contact with the water only on its ventral surface and that the upper lobe does not touch the water at all. Stomata occur in vertical rows on both surfaces of the dorsal lobe and on the upper surface of the ventral lobe

(Demalsy, 1953; Inamdar, 1971). Each stoma has two guard cells initially, though these may fuse to form a single annular guard cell with a central pore (Sud, 1934). Seto and Nasu (1975) reported two guard cells in *A. filiculoides (japonica)* but only one in *A. pinnata (imbricata)*. Inamdar (1971) reported 112 stomata mm<sup>-2</sup> on leaves of *A. pinnata*.

The histology and anatomy of various species of *Azolla* has been studied by a number of workers, both by light microscopy (Campbell, 1893; Sadebeck, 1902; Pfeiffer, 1907; Queva, 1910; Rao, 1936; Smith, 1938; Duncan, 1940; Demalsy, 1953, 1958; Bonnet, 1956, 1957; Sweet and Hills, 1971; Konar and Kapoor, 1972; Walmsley, Breen and Kyle, 1973; Peters and Mayne, 1974a; Fowler, 1975a; Ashton and Walmsley, 1976; Martin, 1976a) and electron microscopy (Kawamatu, 1961, 1962, 1963, 1965a, b; Grilli, 1964; Lang, 1965; Lang and Whitton, 1973; Duckett, Toth and Soni, 1975; Duckett, Prasad and Toth, 1975; Martin, 1976a; Gunning, 1978; Gunning *et al.*, 1976, 1978a, b, c, 1979; Hardham and Gunning 1979).

The stem apex in *Azolla* is upwardly curved and growth is by successive divisions of a three-sided apical cell (Smith, 1938). The central vascular cylinder, which develops from a common mother-cell layer, is considered to be protostelic (Smith, 1938; Sporne, 1962). However, adaptation to an aquatic life has been accompanied by so great a reduction of the vascular tissues that it is impossible to determine this with certainty. Branching is axillary (Demalsy, 1958) and at the base of each branch there is an abscission layer composed of smaller cells (Rao, 1936; Konar and Kapoor, 1972) which facilitates rapid fragmentation and vegetative reproduction. Apart from movement by water currents, the main agents of dispersal of *Azolla* are probably aquatic birds (Smith, 1938; Schofield and Colinvaux, 1969) and man. Adventitious roots arise singly (in fascicles in *A. nilotica* (Demalsy, 1958)) in an acropetal fashion from the common mother-cell layer and have an abscission layer at the point of attachment to the rhizome. Each root bears unicellular root hairs and has a persistent three-layered root cap or sheath which is shed during growth of the basal root hairs (Leavit, 1902; Pfeiffer, 1907; Rao, 1936; Smith, 1938; Duncan, 1940; Kawamatu, 1961). Root length varies from 15 mm (*A. pinnata*) to 120 mm (*A. nilotica*) and in shallow water the roots are able to penetrate into the underlying mud. Kawamatu (1960) recorded

granular mitochondria in the root hairs of *A. pinnata*. Several authors (Rao, 1936; Kawamatu, 1961, 1965a, b; Konar and Kapoor, 1972) have mentioned the presence of chloroplasts in the unicellular root hairs as well as in the cortical layers of the root. Transfer cells have also been detected in the roots (Duckett *et al.*, 1975a, b; Gunning *et al.*, 1978a, b, c).

Leaves arise from the upper cells of the rhizome, a short distance behind the apex. Only the alternate cells on the right and left of the apex develop into leaves resulting in alternate rows of leaves (Rao, 1936). Successive divisions of the leaf-initial cells give rise to upper and lower leaf lobes, no apical cell being produced (Rao, 1936; Smith, 1938). Early in the development of the dorsal lobe, a cavity is formed on the adaxial surface near the leaf base, enclosing a few vegetative cells of the heterocystous blue-green alga *A. azollae* Strasburger which seems to be invariably associated with the stem apex (Pfeiffer, 1907; Rao, 1936; Smith, 1938; Duncan, 1940; Schaede, 1947; Moore, 1969; Walmsley *et al.*, 1973; Ashton and Walmsley, 1976). Further development of the dorsal leaf lobe encloses the algal cells within the cavity, with only a minute opening to the exterior (Smith, 1938; Konar and Kapoor, 1972), which allows gaseous exchange between the cavity and the atmosphere. The cavity is lined with epidermal cells, from which simple or multicellular branched hairs protrude. The newly enclosed algal cells begin to colonize the cavity and become distributed around the periphery of the cavity (Sadebeck, 1902; Rao, 1936; Schaede, 1947; Mechoir and Werdermann, 1954; Shen, 1960; Kawamatu, 1965a, b; Walmsley *et al.*, 1973; Peters and Mayne, 1974a; Hill, 1975; Duckett *et al.*, 1975b; Ashton and Walmsley, 1976). The development of the alga and fern is synchronized. Development of the leaf primordia is accompanied by enlargement of the algal vegetative cells, a few of which differentiate into heterocysts able to fix atmospheric nitrogen. The upper epidermis of the dorsal leaf lobe develops numerous single-celled water-repelling hairs or papillae (Smith, 1938; Shen, 1960).

The *Azolla-Anabaena azollae* association shows distinct features which are a manifestation of the morphology and physiology of the association (Peters, Evans and Toia, 1976). The symbiotic blue-green alga *Anabaena*

*azollae*, together with a few bacteria occur within specialized mucilage-containing chambers in the leaves of the host and are, therefore, not in direct contact with the external environment (Bottomly, 1920; Grilli, 1964; Wieringa, 1968; Peters, 1976). Bottomly (1920) isolated *Pseudomonas* and *Azotobacter* bacteria from *Azolla* leaf cavities. However, Peters and Mayne (1974b) have since shown that the bacteria within the leaf cavity do not fix atmospheric nitrogen.

Recently, Peters (1975, 1976) and Newton (1976) noted that within the leaf cavity, the algal filaments are confined by a limiting membrane-like envelope of unknown composition, covered by a mucilaginous layer. However, earlier studies by Grilli (1964), Lang (1965) and Hill (1975) on the ultrastructure and development of *A. azollae* made no mention of this membrane. Some of the simple and multicellular branched hairs which protrude into the leaf cavities possess the morphological characteristics of transfer cells (Duckett *et al.*, 1975b). Transfer cells have been shown to play an important role in the short-distance transport of solutes in other plant species, e.g. legumes and many aquatic angiosperms (Gunning and Pate, 1969) and may therefore play a similar role in the *Azolla-Anabaena azollae* association. The mucilage within the leaf cavities is of unknown composition (Peters and Mayne, 1974a), possibly secreted by the glandular hairs (Moore, 1969; Konar and Kapoor, 1972). However, Duckett *et al.* (1975a) found that leaf cavities freed of the algal symbiont did not contain mucilage and they speculated that the mucilage was probably derived from the symbiont because mucilage production by blue-green algae is quite usual. This mucilage must facilitate the transfer of solutes between the fern and alga and conversely must retard the rate of diffusion of atmospheric gases to the alga (Duckett *et al.*, 1975a). The development of the leaf cavity with its associated transfer hairs is not dependent upon the presence of *A. azollae* (Peters and Mayne, 1974a; Peters, 1976; Ashton and Walmsley, 1976).

Cytological investigations of three species of *Azolla* indicate that the genus has the smallest chromosomes yet recorded in ferns (Loyal, 1974). Loyal (1958), using a chromosome squash technique, observed the chromosome number of *A. pinnata* to be  $2n = 44$ ; the largest chromosome pair measured only 2,08  $\mu\text{m}$  in length while the smallest measured 1,04  $\mu\text{m}$ . The obser-

vations of Litardiere (1921), that the chromosome number for *A. carolinana* was  $2n = 48$ , were disputed by Loyal (1958) on the basis that "..... observations based on sectioned material may not be trustworthy". Duncan (1940) observed 18 and 20 chromosome pairs in *A. filiculoides*.

#### 4.2.2 Reproductive biology

The life cycle of *Azolla* has been described by numerous authors. Although no one has specifically mentioned methods of inducing sporocarp development, in *A. filiculoides* sporocarp development is known to be associated with mat formation (Talley, Talley and Rains, 1977) and has been recorded during the summer months in temperate regions (West, 1953; Ashton, 1974). However, sporocarp development in *A. pinnata* is associated with winter months in both India (Konar and Kapoor, 1974) and Taiwan (Shen, 1960). Singh (1977a) also mentioned that sporocarp development appeared to retard growth of the fern fronds.

Sporocarp development in *A. carolinana* has been described by Pfeiffer (1907) and Bergad (1972), while Campbell (1893), Sadebeck (1902), Duncan (1940), Smith (1938), Bonnet (1957), Demalsy (1958) and McLean and Ivimey-Cook (1960) described the sporocarps of *A. filiculoides*. Rao (1936), Demalsy (1958), Shen (1960) and Konar and Kapoor (1974) described the sporocarps of *A. pinnata*. The monograph by Demalsy (1953) on *A. nilotica* included much information on its sporocarps, and Lumpkin and Plunknett (1980) note that the sporocarps of *A. microphylla* have been described by the early workers Meyen, Baillon and Morton.

The genus *Azolla* is heterosporous and Rao (1936), Smith (1938) and Bonnet (1957) state that only the lowermost (oldest) leaf of a branch is fertile. Sporocarps, borne on short stalks, arise in pairs (tetrads in *A. nilotica* (Demalsy, 1958)) from the ventral lobe initial, the dorsal lobe forming an involucre. Usually there is a pair of either microsporocarps or megasporocarps, but one of each may be present (Moore, 1969). Microsporocarps are large and globular relative to the smaller ovoid megasporocarps. During the early stages in the development of sporocarps, cells of *A. azollae* (probably from the lowest fertile leaf) become entrapped within the developing indusia (Smith, 1938). The early stages of development are similar in both types of sporocarp, since there is an

elongated receptacle on which numerous mega- and microsporangial initials arise (Bonnet, 1957). However, during the later development of mega- and microsporangia respectively, all the microsporangial initials and some of the megasporangial initials abort in the latter, giving rise to a single megasporangium or numerous microsporangia (Sporne, 1962; Konar and Kapoor, 1974).

The microsporocarp contains 7-130 stalked microsporangia (Duncan, 1940; Svenson, 1944; Godfrey, Reinert and Houk, 1961), each containing 32 or 64 microspores (Svenson, 1944). Within the periplasmodium of each microsporangium, there develop 3-10 alveolar massulae. These are complex, pseudocellular structures in which the microspores are embedded (Martin, 1976a). In living species, the massulae may be eglochidiate (*A. nilotica*), or may have a few hair-like (*A. pinnata*) or fluked (Subgenus *Azolla*) glochidia which serve to anchor each massula to a megaspore ensuring that the micro- and megagametophytes will be held in close proximity to one another (Smith, 1938). Fowler (1975a) has shown that in fossil *Azolla prisca* and extant *A. caroliniana* and *A. filiculoides*, each microspore occupies one of the pseudocellular cavities within the massula. Each microspore-containing cavity is closely associated with the bulbous base of a funnel-shaped cavity, the neck of which extends to the massula's periphery, and which opens by a pore to the exterior. The germinal area of the microspore, marked by a triradiate suture, is always positioned adjacent to the base of the funnel-shaped cavity. Eames (1936), describing microspore germination in modern *Azolla* (i.e. Subgenus *Azolla*), states that a papilla protrudes through the opened suture, then differentiates to form a small prothallus on which an antheridium producing eight spermatozooids develops. Fowler (1975b) considers it likely that the prothallus protrudes into the funnel-shaped exit tube, where the antheridium is developed, and the spermatozooids are released to the exterior via the pore and not by gelatinization of the massula as stated by Smith (1938).

Within the megasporocarp, which takes about a week to mature, is a single megasporangium containing a single megaspore apparatus. The periplasmodium, in which the megaspore initial was originally embedded, gives rise to a thick perispore, analogous to the seed coat in higher plants. The

megaspore apparatus has been described as the most complex megaspore produced by embryophytic plants (Fowler, 1975b; Martin, 1976a). The megaspore apparatus consists of the megaspore, above the proximal pole of which lies a superstructure of hair-like filaments called, by some authors, the columella (Fowler, 1975a). This columella, which is continuous with similar filaments on the megaspore wall (Smith, 1938), bears vacuolate, pseudo-cellular floats. These are analogous to microsporocarpic massulae, and number three in Subgenus *Azolla* or nine in Subgenus *Rhizosperma* (a three float tier above a six float tier). The columella plus floats constitute the so-called 'swimming apparatus', since they were originally thought to have a hydrostatic or insulating function (Bonnet, 1957). However, repeated observations on ripe mega- and microsporocarps after indusial rupture show that they never float but always sink and that gametogenesis, fertilization and the initial development of the sporophyte take place below the water surface (Sud, 1934; Konar and Kapoor, 1974; Ashton, 1977).

Development of a megaspore into a mature gametophyte takes about seven days (Campbell, 1893). The megaspore nucleus divides to form a megagametophyte initial located at the upper end of the megaspore. Following enlargement of this gametophyte initial, the megaspore wall ruptures and the developing gametophyte protrudes through the opening into the columellar space between the floats. Further development of the gametophyte is followed by differentiation of typical archegonia, each containing an egg cell. Fertilization of the egg cell by a spermatozoid results in the formation of a zygote, from which a new sporophyte develops. The developing sporophyte rapidly elongates, displacing the floats and indusium cap, thereby becoming 'infected' at its apex with the akinetes of *A. azollae* present under the indusium cap (Ashton and Walmsley, 1976). As soon as the first leaf appears, the young sporophyte carrying the remains of the megaspore and massulae floats to the surface of the water before developing further.

The continuity of the association between the fern and the alga throughout the life cycle of the fern has been something of an enigma. Benedict (1923), Smith (1938), Shen (1960) and Konar and Kapoor (1974) state that algal cells or spores (akinetes) are found at the apex of both mega- and

microsporangia, whilst Huneke (1933) reported algal akinetes only in the apex of the megasporocarp. On ontogenetic evidence put forward by Smith (1938) and Sporne (1962), algal akinetes must be present within both the mega- and microsporocarps, since their early developmental stages are identical.

#### 4.3 METHODS

##### 4.3.1 Preparation of anatomical sections for light and electron microscopy

Samples of mature *Azolla* plants were collected at several localities in the study area, gently blotted dry and stored on ice in the dark for transport to the laboratory. At the laboratory, the double fixation procedure was modified from the general techniques described by Juniper, Cox, Gilchrist and Williams (1970). Concentrations and fixation times were determined for best overall preservation by experimentation.

##### 4.3.1.1 Symbiotic *Azolla* plants

Whole *Azolla* plants were prefixed in 3 % glutaraldehyde in 0,05 M sodium cacodylate buffer (pH 7,0) for 3 hours at ambient temperature (approximately 22 °C). The samples were then rinsed twice (one hour for each rinse) in buffer at ambient temperature and left overnight in buffer at 4 °C. After this pre-fixation procedure, the samples were post-fixed for two hours with 1 % osmium tetroxide in 0,05 % sodium cacodylate buffer (pH 7,2; 4 °C). This post-fixation was carried out in the dark since light may cause degeneration of osmium tetroxide to black osmic acid (Juniper *et al.*, 1970).

The samples were then thoroughly rinsed with several changes of deionized water, whereafter they were dehydrated through a 20, 40, 60, 80 and 95 % ethanol series, remaining one hour in each concentration, at ambient temperature (approximately 22 °C). Final dehydration took place in absolute ethanol, followed by two 15 minute rinses in absolute propylene oxide.

After dehydration, the samples were carefully cut into short lengths (approximately 3 mm long), infiltrated and embedded in Spurr's soft resin mix (Spurr, 1969) by leaving them overnight at 70 °C in a Reichert KT 100 embedding oven.

Sections (2-4 µm thickness) were cut on an LKB Ultratome III using either a Du Pont diamond knife or glass knives. For electron microscopy, sections were collected on 3,05 mm diameter 300 mesh copper viewing grids and double stained for 20 minutes with a methanol-saturated uranyl acetate solution followed by 20 minutes with basic lead citrate (Reynolds, 1963). The sections were examined and photographed on a Philips EM 301 transmission electron microscope at an accelerating voltage of 40, 60 or 80 kV, depending on the degree of contrast and resolution required. For light microscopy, sections were collected on albumin-treated glass slides, stained with 1 % toluidine blue in 1 % borax, and examined with a Zeiss research photomicroscope.

#### 4.3.1.2 Algal samples

Algal filaments were isolated from *Azolla* leaves or gently removed from agar plates with a spatula, prefixed for 1 hour in 3 % glutaraldehyde in 0,05 M sodium cacodylate buffer (pH 7,0) at ambient temperature and placed in Reichert 'flo-thru' capsules. The filaments were then embedded in a few drops of molten 1,5 % Oxoid No. 3 agar, at 40 °C. Care was taken that the agar was not too hot, as this can affect the fine structure (Reynolds, 1963). After embedding, small cubes of agar (approximately 3 mm sides) containing algal filaments were fixed, stained and sectioned as described above (4.3.1.1).

#### 4.3.2 Measurement of cell development in *Anabaena azollae*

Ten large plants of *A. filiculoides* were dissected under a Wild M5 binocular dissecting microscope, from the base to the apex of the rhizome (i.e. from the oldest to the youngest leaves). Successive leaves were removed to numbered glass phials until the last complete leaf (i.e. leaf cavity closed except for the pore) could be removed. The largest leaf remaining on the undissected portion of the rhizome corresponded to the stage at which the opening to the exterior was closing. This leaf was

considered to be the youngest leaf with algae and was therefore counted as leaf No. 1. The partially developed leaves and rhizome apex were combined and counted as leaf No. 0. Each leaf was then dissected and the colony of algae in the cavity removed with a micromanipulator. The algae were examined unstained in water with a light microscope. Heterocyst frequencies were based on counts of at least 200 cells. The dimensions of vegetative cells were measured with an eyepiece micrometer, at least 40 cells being measured from each leaf. Cell volumes were calculated, assuming the cell shape to be an ellipse (Hill, 1977), using the formula:

$$V = n \cdot w^2 \cdot l / 6 \quad (11)$$

where  $w$  = width,  $l$  = length, and  $n$  = number of cells.

#### 4.3.3 Sample collection and culture methods for life history study

The investigation of the life history of *A. filiculoides* in the study area was divided into three phases: (a) determination of the factors responsible for sporocarp production, (b) study of the developmental stages of megaspore germination, and (c) determination of the factors regulating megaspore germination.

For the first phase, (a), mature plants of *A. filiculoides* were collected in the study area, transported to the laboratory and grown in large constant environment rooms (see Section 2.5.2.1) in a nutrient solution designed to simulate the nutrient conditions occurring beneath an established *Azolla* mat (Table 2). In experiments where a source of combined nitrogen was required, this was supplied as sodium nitrate and ammonium sulphate to give a final concentration of  $1 \text{ mg NO}_3\text{-N} \cdot \ell^{-1}$  and  $1 \text{ mg NH}_4\text{-N} \cdot \ell^{-1}$ , respectively. The effects of various combinations of environmental factors (temperature, pH, light intensity, agitation) were studied and the findings were related to routine physico-chemical and environmental measurements made in the study area.

TABLE 2 - Composition of nutrient solution

Solution <sup>x</sup>	Chemical	Concentration (mg.ℓ <sup>-1</sup> )
1	MgSO <sub>4</sub> ·7H <sub>2</sub> O	250,0
2	CaCl <sub>2</sub> ·2H <sub>2</sub> O	200,0
3	K <sub>2</sub> HPO <sub>4</sub>	20,0
4	Na <sub>2</sub> HPO <sub>4</sub>	20,0
5	H <sub>3</sub> BO <sub>3</sub>	2,0
	MnCl <sub>2</sub> ·4H <sub>2</sub> O	1,5
	ZnSO <sub>4</sub> ·7H <sub>2</sub> O	2,5
	CoCl <sub>2</sub> ·6H <sub>2</sub> O	0,15
	CuSO <sub>4</sub> ·5H <sub>2</sub> O	0,0015
6	(NH <sub>4</sub> ) <sub>6</sub> Mo <sub>7</sub> O <sub>24</sub> ·4H <sub>2</sub> O	0,005
7	FeNa EDTA	15,0

<sup>x</sup>Concentrations were increased one hundred-fold and made up into stock solutions 1-7 as shown. These were diluted when making up the culture solution. (All chemicals used were analytical grade reagents.)

In order to accomplish the second and third phases, (b) and (c), large numbers of megaspores had to be collected from the study area. Preliminary microscopic examination had shown the presence of numerous megaspores in samples of the mud surface collected from the bottom of pools containing thick mats of *A. filiculoides*. Mud samples were therefore collected by hand, transported to the laboratory, placed in a 250 µm mesh sieve and cleaned by gentle washing in a stream of tap water. The residue in the sieve was examined with a Wild M5 binocular dissecting microscope and megaspores removed with forceps to 450 ml plastic dishes containing 200 ml nutrient solution (Table 2).

Preliminary experimentation had shown that high light intensities reduced the percentage megaspore germination, so all dishes were painted with matt black paint. Fifty megaspores with attached massulae were placed in each dish before being placed in the constant environment rooms. Each

experiment was repeated three times and each experimental treatment was replicated six times. Sporocarp production was expressed as the percentage of plants bearing sporocarps.

The developmental stages in germination were examined with a Wild M5 dissecting microscope and successive stages were drawn with the aid of a camera lucida attachment. Attempts to photograph the various stages of development were unsuccessful due to the shallow depth of focus and the presence of numerous air bubbles.

#### 4.4 RESULTS

##### 4.4.1 Morphology and anatomy of *Azolla filiculoides* from the study area

Plants of *A. filiculoides* from the study area were generally elongated in shape, often 20-40 mm in length (Frontispiece). The leaves were imbricate, papillose and oblong in shape, some 1-1,5 mm in length and closely appressed to the rhizome. Plants growing in shaded areas appeared yellowish-green with a darker green central spot on the dorsal lobe. This dark green spot was due to the colony of *Anabaena azollae* in the cavity of the dorsal lobe. Those plants growing in full sunlight were a deep reddish colour and the darker central spot was not visible. Plants from the lower layers of established mats became chlorotic and died. When growing luxuriantly, the fronds of each plant became slightly curled.

The dorsal lobe of each leaf is considerably thicker than the ventral lobe which is often only 2-3 cells thick. The basal portion of both dorsal and ventral lobes have extensive aerenchymatous tissue which must assist flotation (Plate 15a). The dorsal lobe has a well-developed palisade layer and the epidermal cells extend out from the leaf margins to form a conspicuous hyaline margin consisting of 3-4 rows of cells (Plate 15c, d). Some of the epidermal cells on the upper surface of the dorsal lobe become elongated to form uni-cellular papillae (Plate 15c). Stomata occur on both surfaces of the dorsal lobe and on the upper surface of the basal portion of the ventral lobe. Each stoma has two guard cells (Plate 15e). The apical region of the rhizome bears numerous colonies of *A. azollae* (Plate 15b) which enter the cavities of newly-formed leaves (Plate 16a).

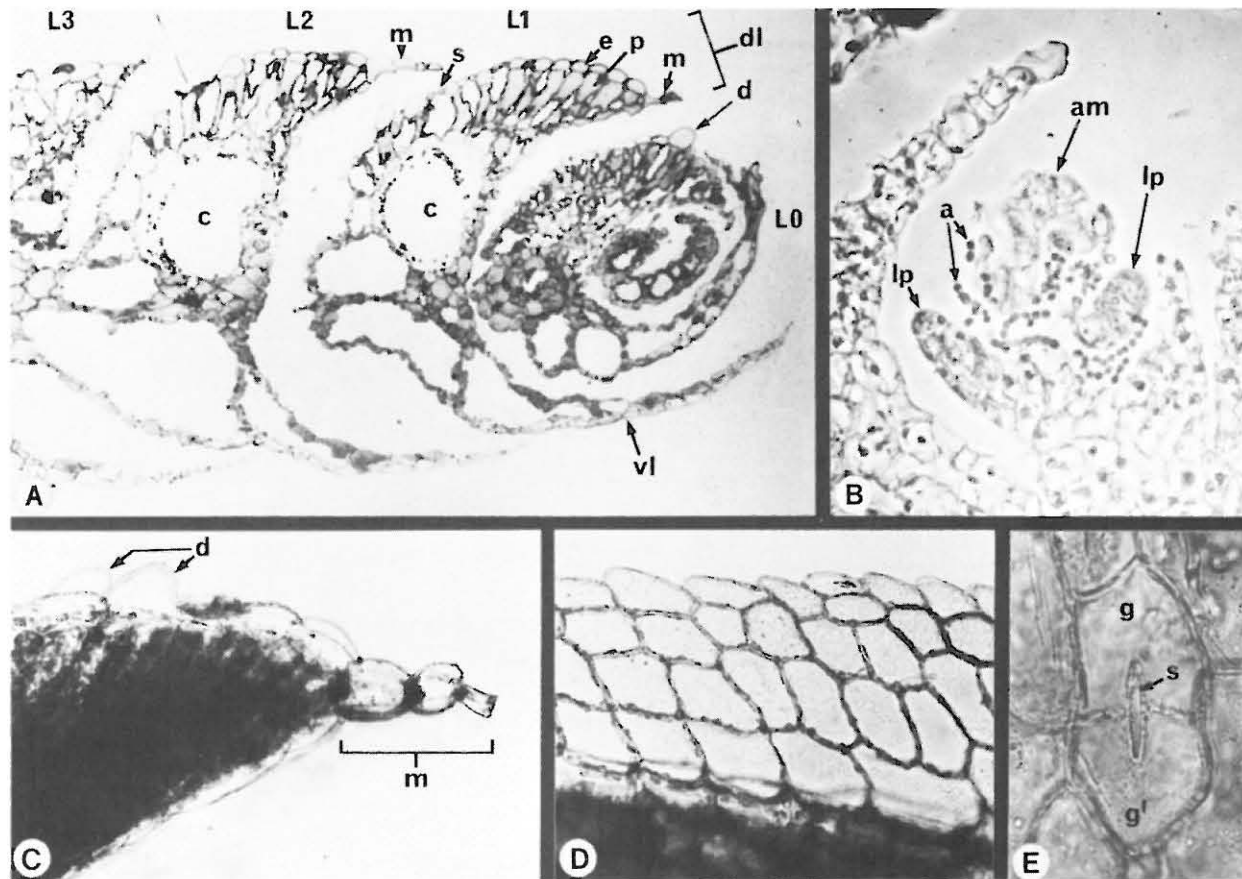


PLATE 15 : Morphology and anatomy of *Azolla filiculoides* Lam. (1).

A (X30) LS of branch apex showing apical region (L0) and first, second and third leaves (L1, L2, L3). Each leaf has a ventral (vl) and dorsal lobe (dl). Each dorsal lobe contains a cavity (c) with filaments of *Anabaena azollae*, and has a thick pallisade layer (p). The epidermis (e) is one cell thick on the leaf margin (m) with scattered papillae (d) and stomata (s). B (X100) Apex of *Azolla* branch, showing apical meristem (am), leaf primordia (lp) and associated algal cells (a). C (X125) Lateral view of leaf showing one-cell thick margin (m) and dorsal papillae (d). D (X125) Dorsal view of leaf margin. E (X300) Dorsal leaf-lobe stoma (s) with two guard cells (g, g').

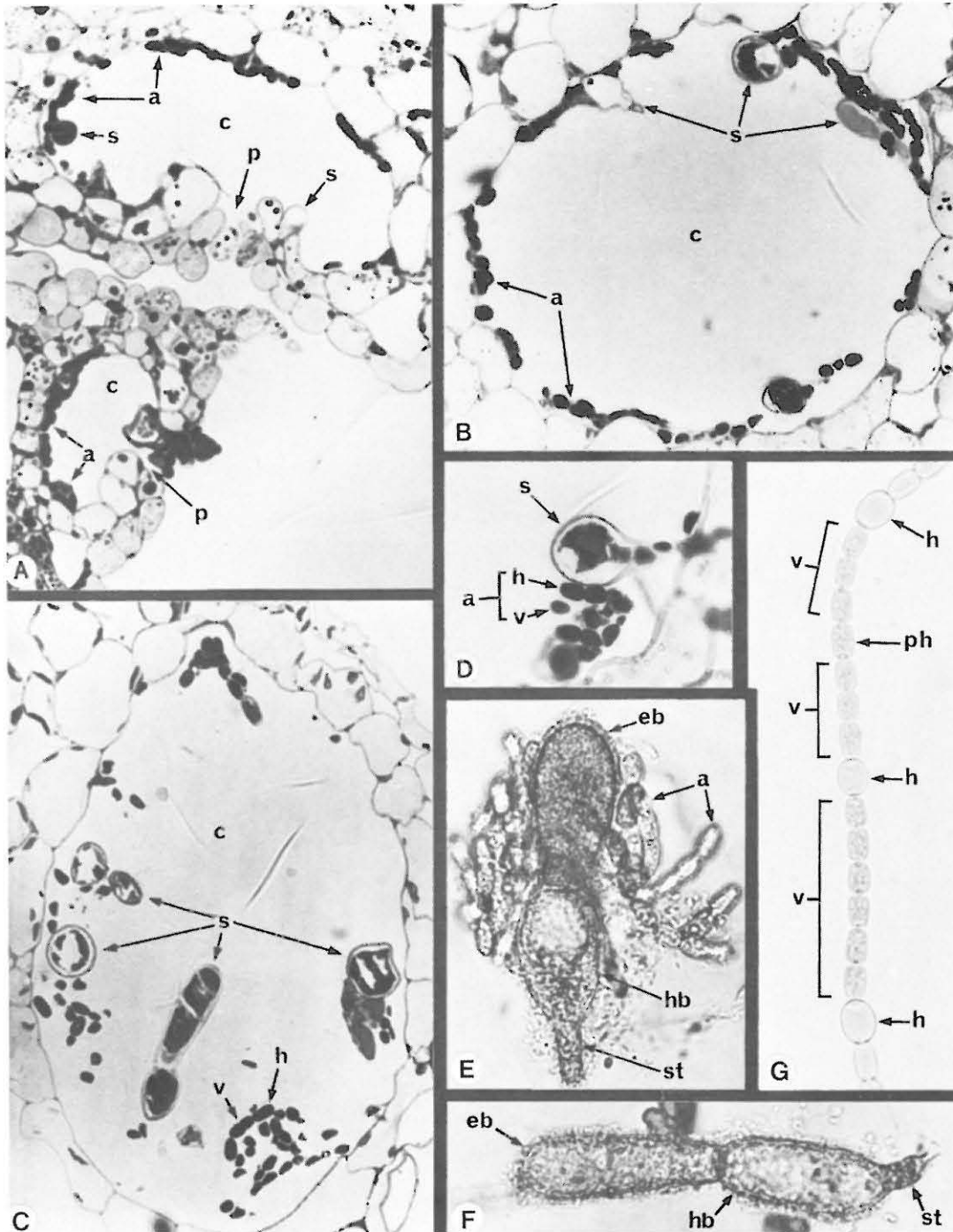


PLATE 16 : Morphology and anatomy of *Azolla filiculoides* Lam. (2).

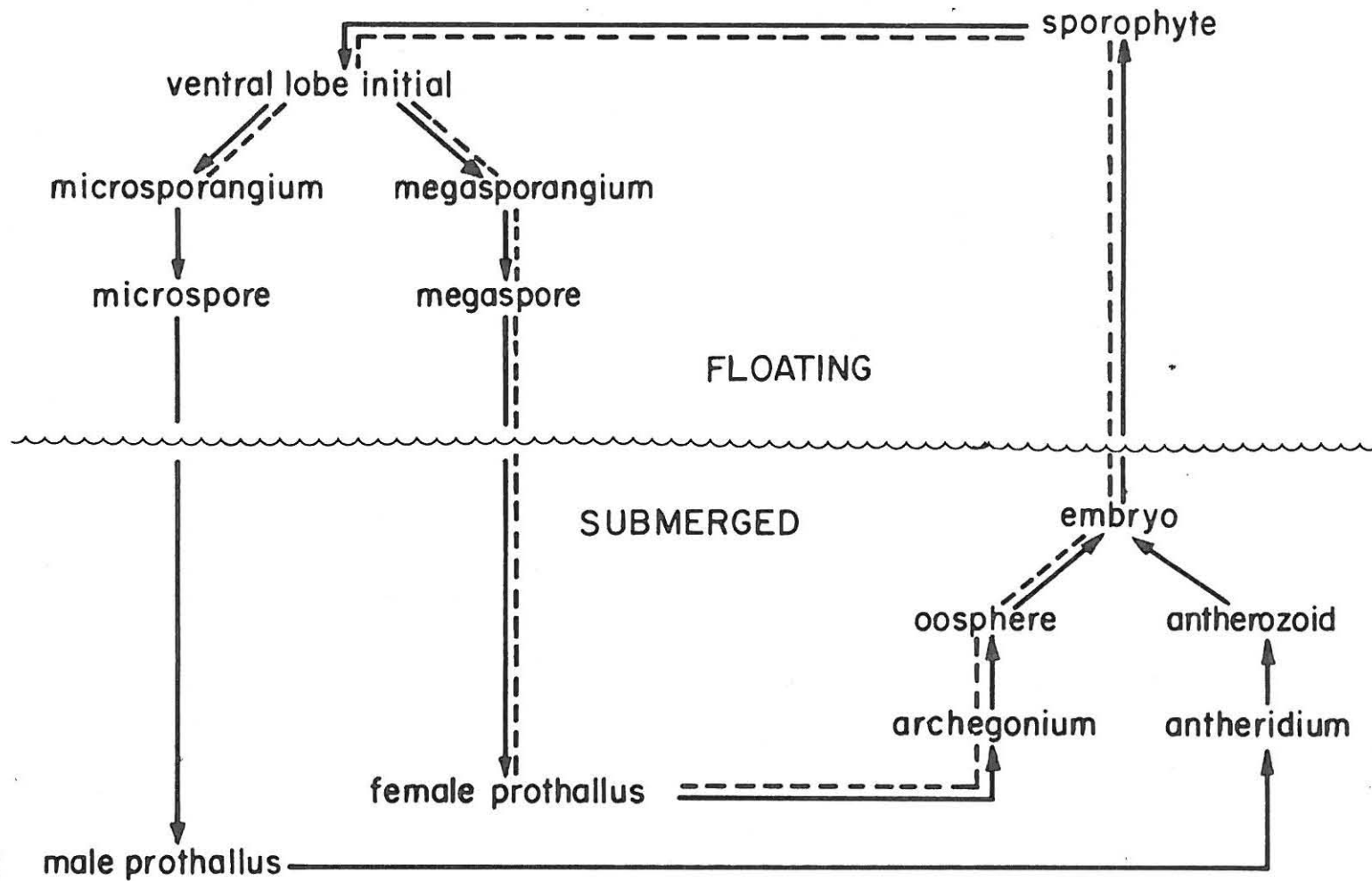
A (X275) Developing leaf cavities (c) of *A. filiculoides*, showing pores (p), secretory hair cells (s) and filaments of *Anabaena azollae* (a). B (X325) Section of leaf cavity (c) in young leaf, showing filaments of *A. azollae* (a) and small secretory hair cells (s). C (X375) Section of leaf cavity (c) in older leaf, showing differentiated vegetative cells (v) and heterocysts (h) of *A. azollae* dispersed among large secretory hair cells (s). D (X375) Young secretory hair cell (s), showing close association with heterocysts (h) and vegetative cells (v) of *A. azollae* (a). E (X550) Secretory hair cell consisting of epibasal (eb), hypobasal (hb) and stalk (st) cells, and associated filaments of *A. azollae* (a). F (X550) Secretory hair cell with algal filaments removed, showing epibasal (eb), hypobasal (hb) and stalk (st) cells. G (X625) Filament of *A. azollae* showing vegetative cells (v), heterocysts (h) and developing proheterocyst (ph).

Initially, the algal filaments are closely appressed to the walls of the cavity (Plate 16b), but in older leaves the algal colonies become more dispersed (Plate 16c). Secretory hair cells, arising from the epidermal cells lining the cavity, protrude into the cavity. In newly-developed leaves, these secretory hairs are often single-celled (Plate 16d), but in older leaves each secretory hair becomes multicellular (Plate 16c, e, f). Each secretory hair consists of an epibasal, a hypobasal and a stalk cell (Plate 16e, f). Under the light microscope, the cell contents appear highly granular. Numerous bacteria can be seen adhering to the exterior surface of the hair cells (Figure 16f). Within each leaf cavity, algal filaments become closely associated with these secretory hairs.

In mature plants of *A. filiculoides* from the study area, the adventitious roots were almost always simple (lacking root hairs) and approximately 50 mm in length. Each root has an abscission layer at its point of attachment to the rhizome and a root cap, 5-7 mm long, which was sloughed off when the root had attained its full length.

In this study observations showed that the life cycle stages of *A. filiculoides* corresponded closely to those of other heterosporous ferns, as described by Sporne (1962). These stages of development are summarized in Figure 9.

Pairs of sporocarps were borne by short stalks at the base of the first ventral lobe initial of a lateral branch, the dorsal lobe forming an involucre. Usually there was one sporocarp of each sex present, though occasionally, pairs of megasporocarps or microsporocarps were found. Each microsporocarp contained numerous stalked microsporangia. In this study 50 microsporocarps were examined and found to contain an average of 73 microsporangia (range: 24-117). The microsporangia were examined and found to contain, on average, 7 massulae, each massula bore aseptate (or very rarely uniseptate), fluked glochidia (Plate 14). Field observations showed that, on rupture of the microsporocarp, the microsporangia sank to the bottom of the water body, ruptured and released the massulae.



(The line (----) shows the continuation of the association with *Anabaena azollae* ).

Fig.9 *Heterosporous life cycle of Azolla filiculoides* Lam.

The ovoid megasporocarps were found to be much smaller than the globular microsporocarps. Within each megasporocarp a single megasporangium, containing a single megaspore, was found. The distal portion of the megasporangium wall remained attached to the megaspore, after rupture of the megasporocarp, and formed an indusium cap. Within the indusium cap was the so-called 'swimming apparatus' consisting of 3 pseudocellular floats. After rupture of the megasporocarp, the megaspore sank to the bottom of the water body and gametogenesis, fertilization and the initial development of the sporophyte took place below the water surface. The young sporophytes only floated to the water surface after development of two to three leaves.

#### 4.4.2 Maintenance of the fern-alga association

Throughout the vegetative growth of the *Azolla* sporophyte, developing leaf primordia at the stem apex trapped cells of *A. azollae* within cavities in the dorsal leaf lobes (Plate 15). These algal cells then enlarged and differentiated as the *Azolla* leaf aged (Plates 15 and 16). During sporocarp formation, algal cells were found within the indusia of both mega- and microsporocarps. Liberation of mature microsporangia by rupture of the microsporocarp dissipated all the algal cells originally trapped within the microsporocarp indusium. On the other hand, liberated mature megaspores retained the indusium cap and therefore the algal cells trapped beneath it. After fertilization of the megaspore, the developing zygote elongated (Figure 10), the stem becoming 'infected' at its apex with the cells of *A. azollae*. With the formation of the first and second leaves, the young sporophyte floated to the surface of the water before developing further. This pattern is summarized in Figure 9.

#### 4.4.3 The developmental stages of megaspore germination

Field observations showed that megaspore germination took place on the surface of the mud at the bottom of the water body, often in very shallow (0,1-0,2 m) areas. In the laboratory megaspores germinated both in culture solutions and in mud samples placed on a laboratory window sill. Germination (the appearance of the first leaf) took from 17 to 43 days after artificial combination of megaspores and microspore-containing massulae. When collected in the field, each megaspore bore clusters of

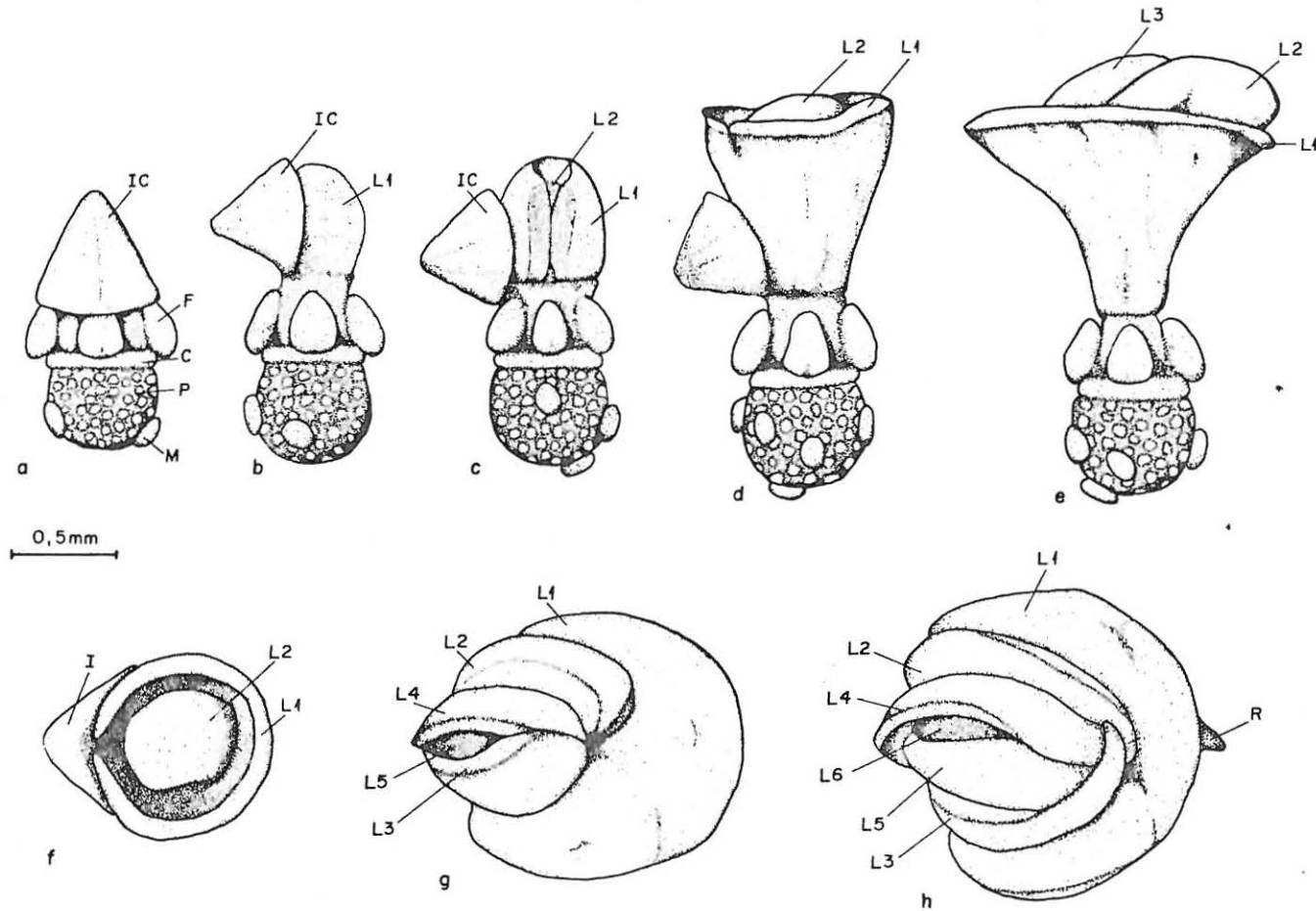


Fig. 10 *Developmental stages in the germination of an Azolla filiculoides megaspore. (a-e) lateral view, (f-h) dorsal view; [C = collar, F = "float", IC = indusium cap, M = attached massula, P = patterned perispore, R = first root, L1, L2---L6 = first, second---sixth leaf]. (f and g correspond to dorsal views of d and e respectively).*

massulae, attached to the minute matted hairs of the perispore wall by their fluked glochidia. In many cases the attached massulae obscured detail of the perispore but these were removed before the different germination stages were drawn (Figure 10).

The first visible sign of germination was the upward displacement of the indusium cap, revealing the floats, due to expansion of the first leaf within the central columellar space (Figure 10a). This was followed by lateral displacement of the indusium cap, which remained attached to the base of the columella by the enlarging first leaf (Figure 10b). In its initial stages, this first leaf was almost transparent and somewhat cylindrical in shape. The first leaf enlarged to form a hollow cylinder, in the middle of which the second leaf could be seen (Figure 10c). At this stage a constriction between the base of the first leaf and the columella was evident. The second leaf was also cylindrical in shape initially and enlarged in unison with the first leaf (Figure 10d, f). The first leaf continued to enlarge, becoming flattened and semi-circular when viewed from above (Figure 10e, g). The second leaf, now much smaller than the first leaf, was laterally flattened and displaced by the third and successive leaves. These leaves were produced alternately and were closely appressed to each other (Figure 10g, h). During this process, the constriction between the base of the first leaf and the columella became more pronounced and the indusium cap was lost. At the second leaf stage, i.e. Figure 10d, the new sporophyte floated to the water surface where further development took place. The first root appeared at the base of the first leaf and the remains of the old megaspore broke away from the sporophyte at the abscission layer produced at the constriction point between the base of the first leaf and the columella (Figure 10h). Continued development resulted in rapid elongation of the rhizome and the production of further leaves and roots.

#### 4.4.4 Cell development in *Anabaena azollae*

Vegetative cells of *A. azollae* showed a definite sequential development, becoming larger and changing colour from light green to yellowish green with increasing distance from the shoot apex (Figures 11 and 12). At the shoot apex, the algal cells appeared to be in frequent cell division,

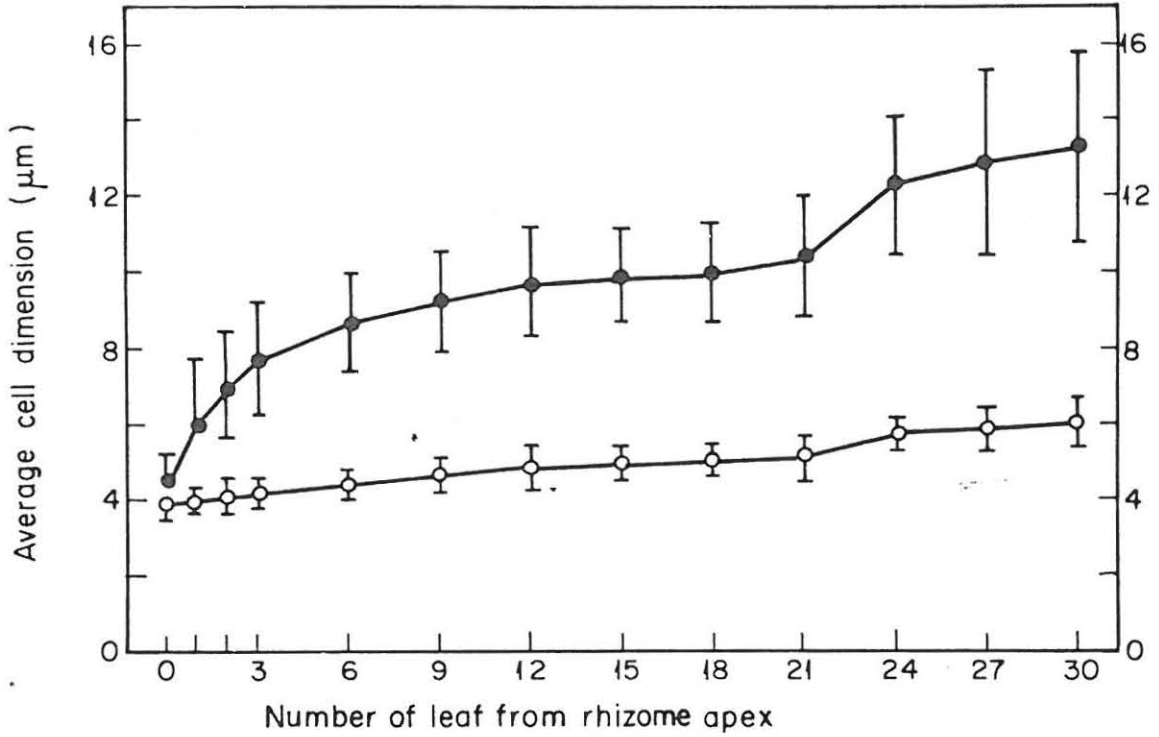


Fig. 11 *Variation in size of vegetative algal cells in successive leaves from the apex. ○ = width; ● = length. (Vertical lines indicate standard error of means).*

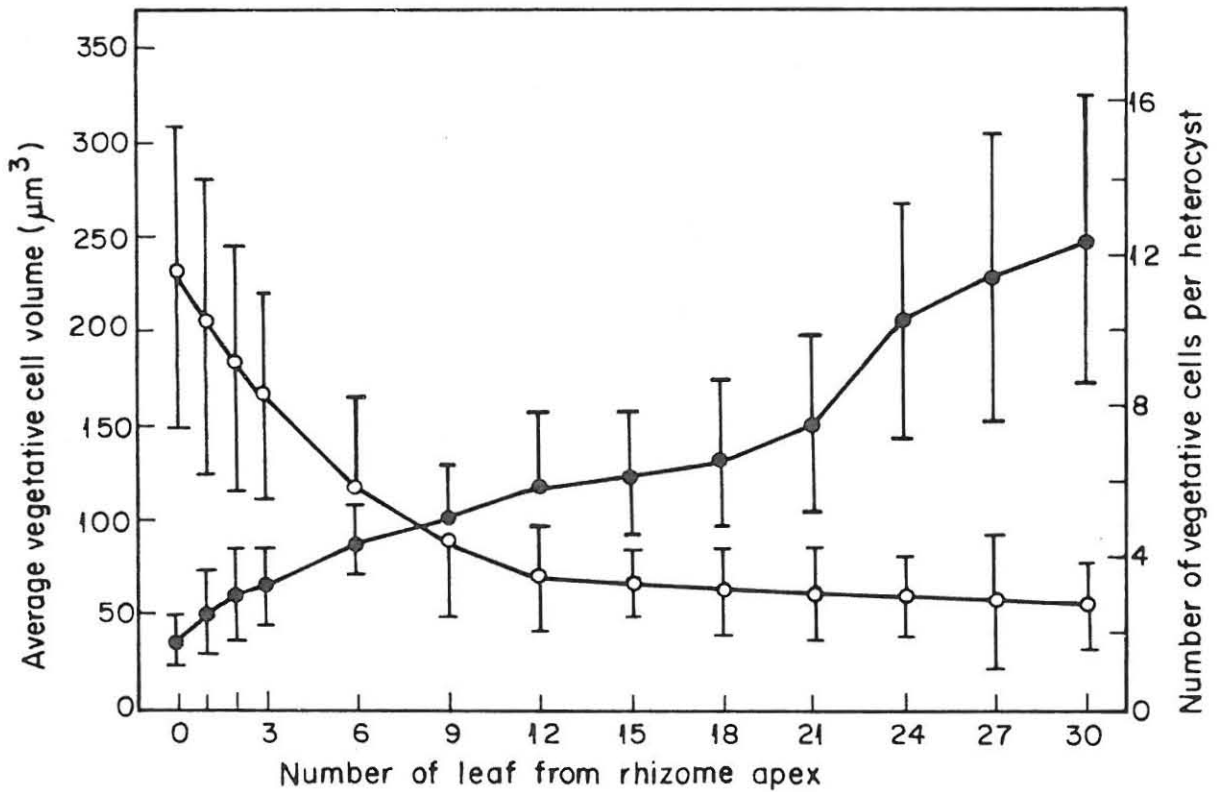


Fig. 12 *Volume of vegetative cells and number of vegetative cells per heterocyst of the alga in successive leaves from the apex. ● = volume; ○ = vegetative cells per heterocyst. (Vertical lines indicate standard error of means)*

while further back cell division became less common although there was considerable variation between filaments. Observations showed an initial rapid increase in vegetative cell length to about leaf 6, followed by a gradual increase up to leaf 21. This was followed by a second phase of rapidly increasing cell length up to leaf 24 and a further gradual increase to leaf 30 (Figure 11). Changes in the width of vegetative cells followed a similar, though less marked, pattern. From the apex to senescent leaves, the vegetative cells increased in width from  $3,9 \mu\text{m} \pm 0,6$  to  $6,1 \mu\text{m} \pm 0,8$ ; i.e. an average of  $\times 1,6$ . In the same leaves, vegetative cells increased in length from  $4,5 \mu\text{m} \pm 0,7$  to  $13,1 \mu\text{m} \pm 2,4$ ; i.e. an average of  $\times 2,9$ . The volume increased, on average, about  $\times 7,1$  over the shoot length, but this was very variable due to the multiplied variation of length and width (Figure 12). In older leaves (24-30) the vegetative algal cells appeared to be enlarged and senescent, losing their colour and cellular contents and becoming clumped in masses or short chains. Vegetative algal cells in mature leaves (6-21), appeared to be still functional, retaining their colour and remaining in long filaments.

Counts of heterocyst frequency were complicated by the difficulty in distinguishing between developing heterocysts ('proheterocysts', see Plate 16g) and mature heterocysts, particularly in the apical leaves. In this study heterocysts were counted if they were enlarged cells of rounder shape and had thick cell walls, polar bodies, a yellowish colour and apparently homogenous cell contents or a combination of most of these characteristics. In the older *Azolla* leaves (i.e. leaves 21-30) senescent algal cells and the presence of numerous akinetes also complicated heterocyst counts. With increasing leaf age, the frequency of heterocysts (expressed as a percentage of all algal cells) increased from  $8,1 \% \pm 2,8$  to a maximum of  $32,5 \% \pm 1,9$  at about leaf 18 and then remained more or less constant to leaf 30 (Figure 12).

The varying proportions of vegetative cells, heterocysts and akinetes with increasing leaf age, expressed as a percentage of all algal cells, are shown in Figure 13. There is considerable variation between different plants, though the trends remain the same. With the increase in heterocyst frequency from the apex to leaf 15, the proportion of

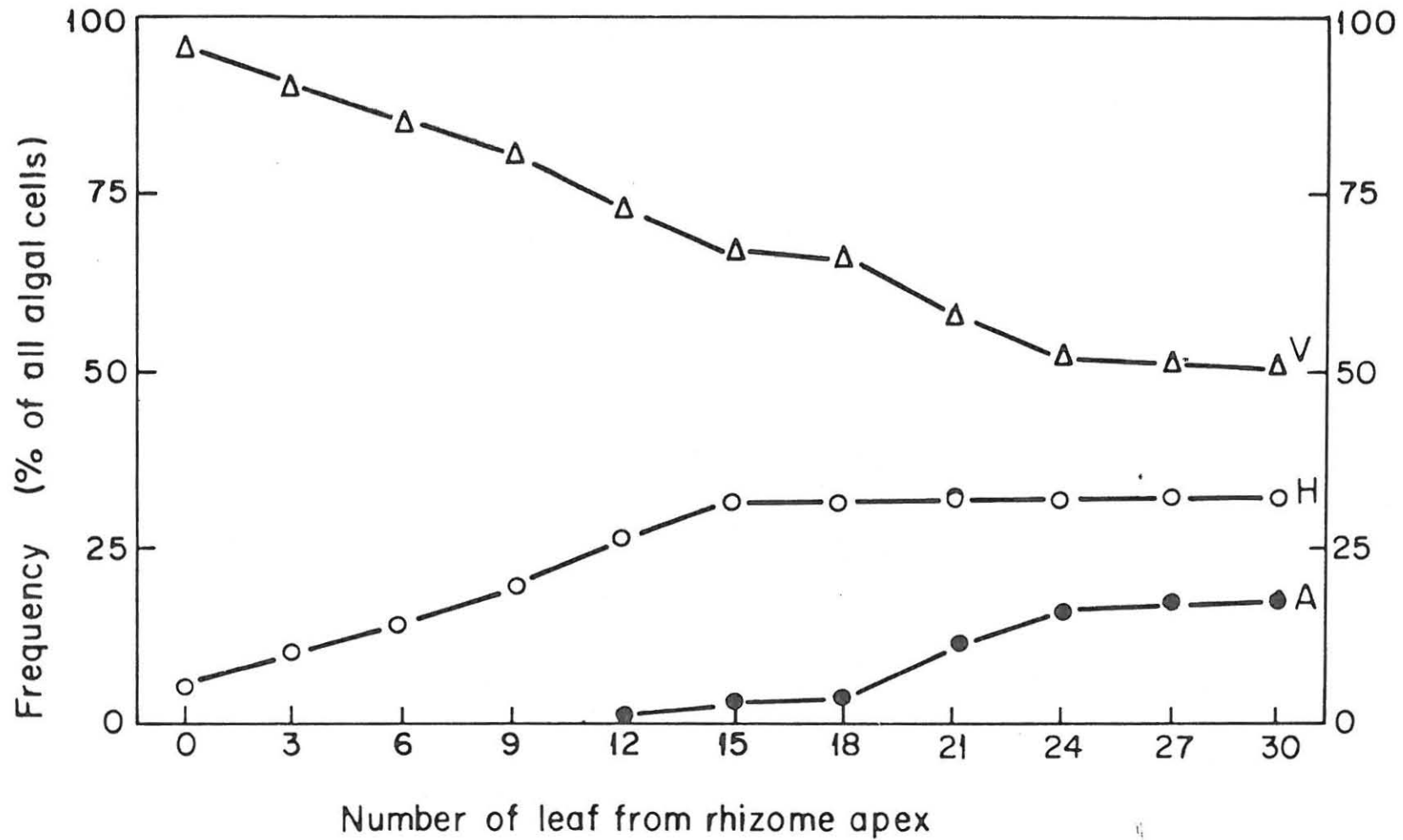


Fig. 13. *Frequencies of vegetative cells (V), heterocysts (H) and akinetes (A) in successive leaves from the shoot apex of A. filiculoides.*

vegetative cells decreased. As the akinete frequency increased from leaf 15 to leaf 24, the proportion of vegetative cells decreased to a minimum. From leaves 24 to 30 the proportions of the three cell types remained almost constant.

At the ultrastructural level, the three cell types viz. vegetative cells, heterocysts and akinetes, were examined both in the intact *Azolla-Anabaena* symbiosis and in cultures of isolated *A. azollae*. The main ultrastructural features are shown in Plates 17-20.

Each vegetative cell possesses a 4-layered cell wall which is visible in the electron micrograph (Plate 17) as a multi-layered structure. Outside this cell wall a narrow sheath is partly visible. This sheath is not normally seen in symbiotic material under the light microscope and is often reported as being absent (Grilli, 1964; Lang, 1965). Within the 4-layered cell wall is a thin plasma membrane or plasmalemma which maintains the physiological integrity of the cell. The most extensive sub-cellular structures seen with the electron microscope are the flattened vesicles ('thylakoids'; Menke, 1961), which ramify throughout the outer regions of the cell. In young cells the thylakoids run mainly parallel to the cell wall (Plate 18), while in older cells and heterocysts, the thylakoids become distributed throughout the cytoplasm or central nucleoplasmic region (Plates 17, 19). In ageing cells and heterocysts, the normally narrow inter-thylakoidal spaces often appear dilated (Plate 19).

Several cytoplasmic inclusions were found in *A. azollae*. Polyglucan granules (' $\alpha$  granules'; Pankratz and Bowen, 1963) were observed in the spaces between plates of thylakoid membranes. Spherical osmiophilic granules, resembling the lipid droplets of chloroplasts (Lang, 1965) are scattered among the thylakoids, but are more frequent near the cell surface (Plates 18, 19). Large conspicuous electron-dense granules with an apparently fibrillar structure ('structured granules'; Drews and Niklowitz, 1956; 'cyanophycin granules'; Fogg *et al.*, 1973) are absent or sparse in very young cells (Plate 18) but are abundant in older cells and akinetes (Plates 17 and 20).

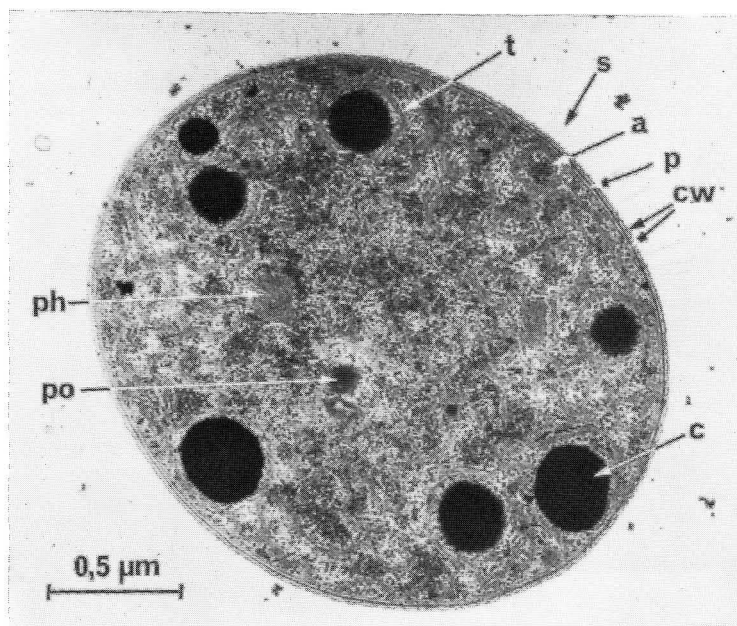


PLATE 17: Ultrastructure of *Anabaena azollae* (1): Strain RU/PA 6a.

Oblique cross section of vegetative cell shows sheath (s), 4-layered cell wall (cw), plasmalemma (p),  $\alpha$  granules (a), thylakoids (t), electron dense cyanophycin granules (c), polyhedral body (ph) and polyphosphate body (po).

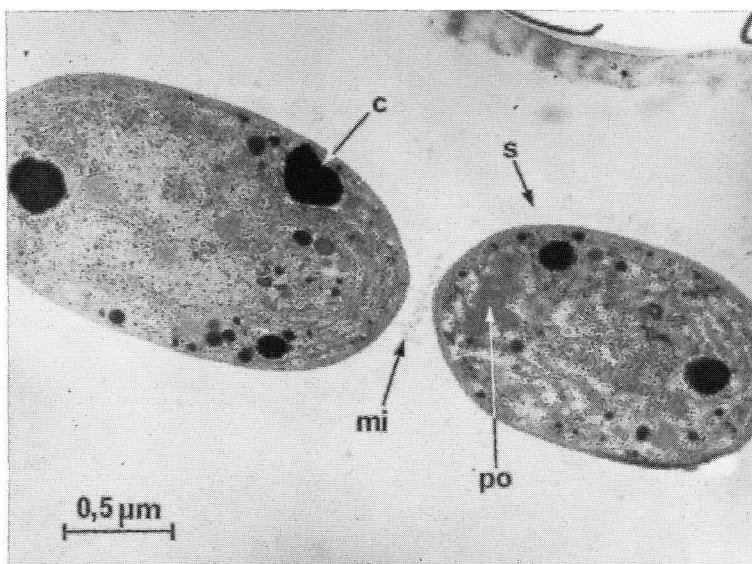


PLATE 18: Ultrastructure of *Anabaena azollae* (2): Symbiotic cells in *Azolla filiculoides*.

LS of junction between two vegetative cells showing sheath (s) and cyanophycin granules (c). Unknown microbodies (mi) can be seen between the two cells. (Note reduced number of cyanophycin granules and increased polyphosphate bodies (po) compared with Plate 17.)

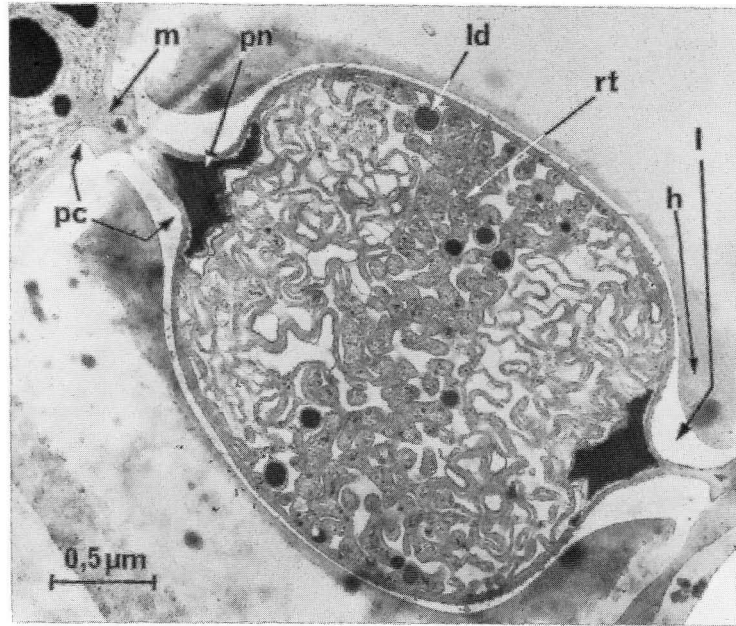


PLATE 19: Ultrastructure of *Anabaena azollae* (3): Strain RU/PA 6a.

LS of intercalary heterocyst showing thickened envelope composed of homogenous (h) and laminated (l) layers, with polar modules (pn), microplasmadesmata (m) and pore channel (pc). Within the heterocyst, lipid droplets (ld) are interspersed with reticulate thylakoids (rt).

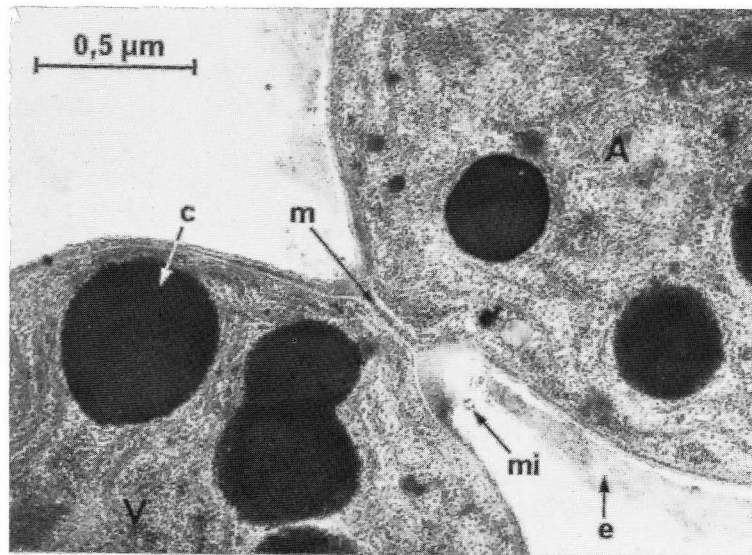


PLATE 20: Ultrastructure of *Anabaena azollae* (4): Symbiotic cells in *Azolla filiculoides*.

LS of junction between akinete (A) and vegetative cell (V), showing microplasmadesmata (m) and cyanophycin granules (c). Akinete has thickened envelope (e) and unknown microbodies (mi) are visible near the junction of the two cell types.

Polyphosphate bodies ('metachromatic granules' or 'volutin granules'; Fritsch, 1945) are electron-opaque bodies which serve as stores of accumulated polyphosphate (Fogg *et al.*, 1973). In young cells, these structures were small and scarce but became more prominent in older cells (Plate 17) and appeared to be absent from heterocysts and akinetes (Plate 19).

In the fibrous nucleoplasmic region, large (200-300 nm diameter) electron-opaque, cytoplasmic inclusions with a distinct polygonal profile are also found (Plate 17). These are possibly the 'polyhedral bodies' of Jensen and Bowen (1971).

During the process of differentiation from a vegetative cell, the heterocyst develops a characteristic ultrastructure (Lang, 1965). A prominent feature is the thick envelope which is laid down outside the 4-layered cell wall and which extends over the heterocyst except at the polar regions (Plate 19). This envelope usually has three distinct layers (Fogg *et al.*, 1973): (a) an outermost loose fibrous layer of irregular thickness, (b) a broad and homogenous middle layer which is particularly well-developed about the pore channel (Plate 19), and (c) a laminated innermost layer which is thick around the pore channel and which thins out towards the median region (Plate 19). The intercellular connection between the heterocyst and adjacent vegetative cells is restricted to a small area of septum across the narrow pore channel between the two cells. Fine connections across the septum (microplasmodesmata) connect the plasma membranes of the heterocyst and vegetative cell (Plate 19). Similar microplasmodesmata occur at the junction of akinetes and vegetative cells (Plate 20).

Within differentiated heterocysts, the nucleoplasmic region is less conspicuous. The typical arrangement of the photosynthetic thylakoids is replaced by a reticulate lamellar system, with concentration of the lamellae towards the polar regions and an accumulation of lipid droplets in those lamellae lying in the median region (Plate 19). Within the pore channel, electron-dense plugs ('polar nodules'; Lang and Fay, 1971) occur. The small, ill-defined 'microbodies' seen near the junctions between adjacent cells, may in fact be artefacts of the fixation and staining procedures.

The mature akinete retains a nucleoplasmic region, photosynthetic thylakoids, polyhedral bodies and cyanophycin granules (Plate 20), but polyphosphate bodies are no longer visible. A dense amorphous envelope surrounds the akinete (Plate 20).

#### 4.4.5 Factors responsible for sporocarp production

In the study area *A. filiculoides* normally produced sporocarps during the summer, from late September to February, and germinated sporelings of *Azolla* could be found from mid-December to early April. The production of sporocarps is an important part of the *A. filiculoides* life cycle and it was considered important to understand the mechanisms regulating sporocarp production.

Field observations showed that mats of *A. filiculoides*, growing in streams which received a continuous inflow of water, initiated sporocarp production in September, 3 to 4 weeks earlier than mats of *A. filiculoides* growing on farm dams receiving intermittent inflows. These two habitats showed marked differences in pH and nutrient status and were examined in detail. At the same time, laboratory experiments were carried out to assess the effects of photoperiod, light intensity, temperature, pH and nitrogen supply on sporocarp production in *A. filiculoides*.

##### 4.4.5.1 Laboratory experiments

###### (a) Photoperiod

The effects of increasing photoperiod on the relative growth rate and sporocarp production of *A. filiculoides* are shown in Figure 14. The relative growth rate increased rapidly with increasing photoperiod and reached a maximum of 0,18 at a 16-hour photoperiod. In contrast, sporocarp production in *A. filiculoides* was not initiated at photoperiods shorter than 8 hours. With increasing photoperiod, the percentage of plants bearing sporocarps increased until a maximum (40 %) was recorded at a photoperiod of 14 hours. Further increases in photoperiod did not increase sporocarp production.

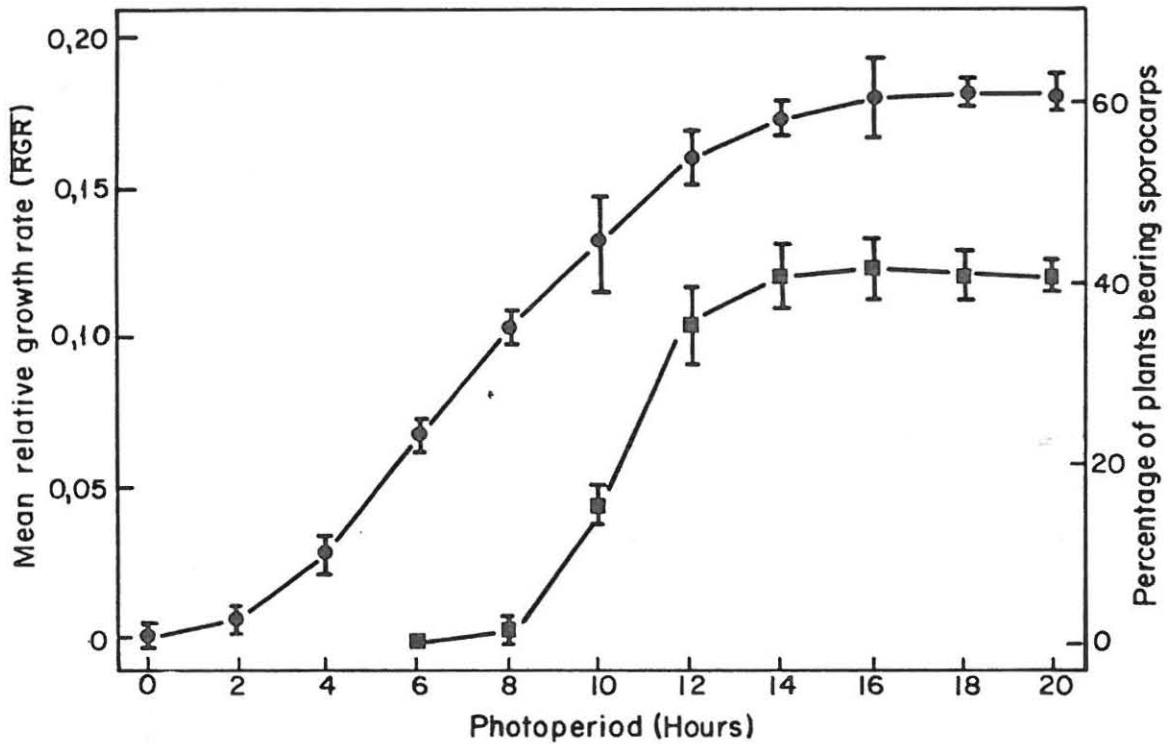


Fig. 14 *The relative growth rate (RGR) (●—●) and percentage of Azolla filiculoides plants bearing sporocarps (■—■) after 40 days growth under different photoperiods. (Vertical bars indicate 95% confidence limits). [30kLux; pH 9,5; 27,5 °C].*

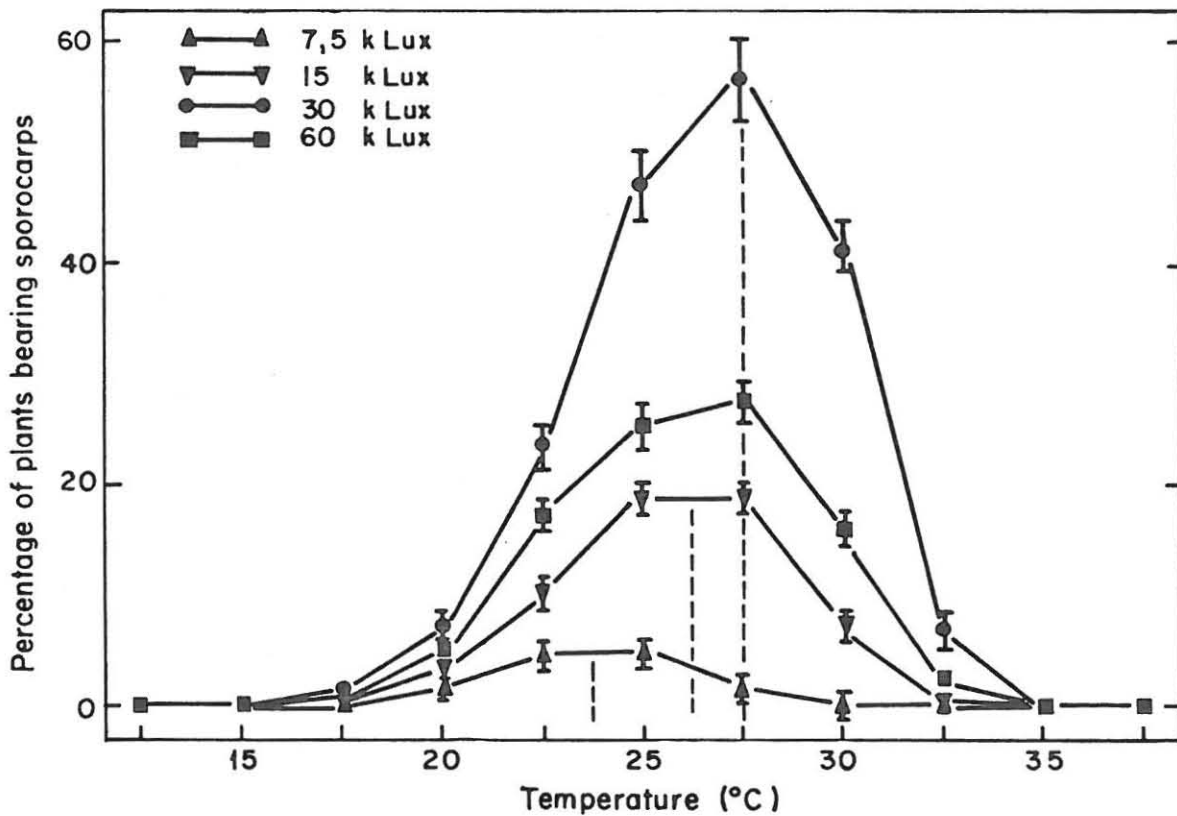


Fig. 15 *The effects of temperature and light intensity on the number of Azolla filiculoides plants bearing sporocarps. (Vertical bars indicate 95% confidence limits). [14 hour day; pH 9,5].*

(b) Light intensity and temperature

Light intensity and temperature were found to have interacting effects on sporocarp production under optimal conditions of pH and photoperiod (Figure 15). No sporocarps were produced at temperatures below 17,5 °C and above 35 °C at any of the light intensities used. However, within the range 20 °C to 30 °C the percentage of plants bearing sporocarps increased with increasing light intensity from 7,5 kLux to 30 kLux. High light intensity (60 kLux) led to a decrease in the percentage of plants bearing sporocarps over the whole temperature range used. An interesting feature of these results is that the 'optimum' temperature for sporocarp production increased with increasing light intensity, to a maximum (27,5 °C) at 30 kLux.

(c) Temperature and pH

The effects of pH on the sporocarp production of *A. filiculoides* plants grown at three different temperatures are shown in Figure 16. At the 'optimum' temperature of 27 °C sporocarp production was highest in the pH range 8,5 to 10,0 with a maximum at pH 9,5. Sporocarp production was considerably reduced at pH values below 8,5 or above 10,0 and also at temperatures above 27 °C or below 22 °C, over the whole pH range.

(d) Nitrogen source

Previous work by Ashton (1974) showed that as the plant density per unit area increased during mat formation, the rate of nitrogen fixation (ethylene production) decreased to a very low level. However, even under favourable environmental conditions in the laboratory, *A. filiculoides* only formed sporocarps when the plants were in an established mat and the rate of vegetative growth had decreased to a minimum (Table 3). The low rates of nitrogen fixation at this stage were insufficient to maintain the nitrogen content of the fern-alga association and thus the nitrogen requirements of the association must have been partly fulfilled by the uptake of a source of combined nitrogen from the underlying waters. The effect of nitrate concentration on sporocarp production at three different temperatures was therefore investigated and it was observed that sporocarp production increased with increasing nitrate concentration to a maximum at 1 mg.l<sup>-1</sup> NO<sub>3</sub>-N (Figure 17). A nitrate concentration of

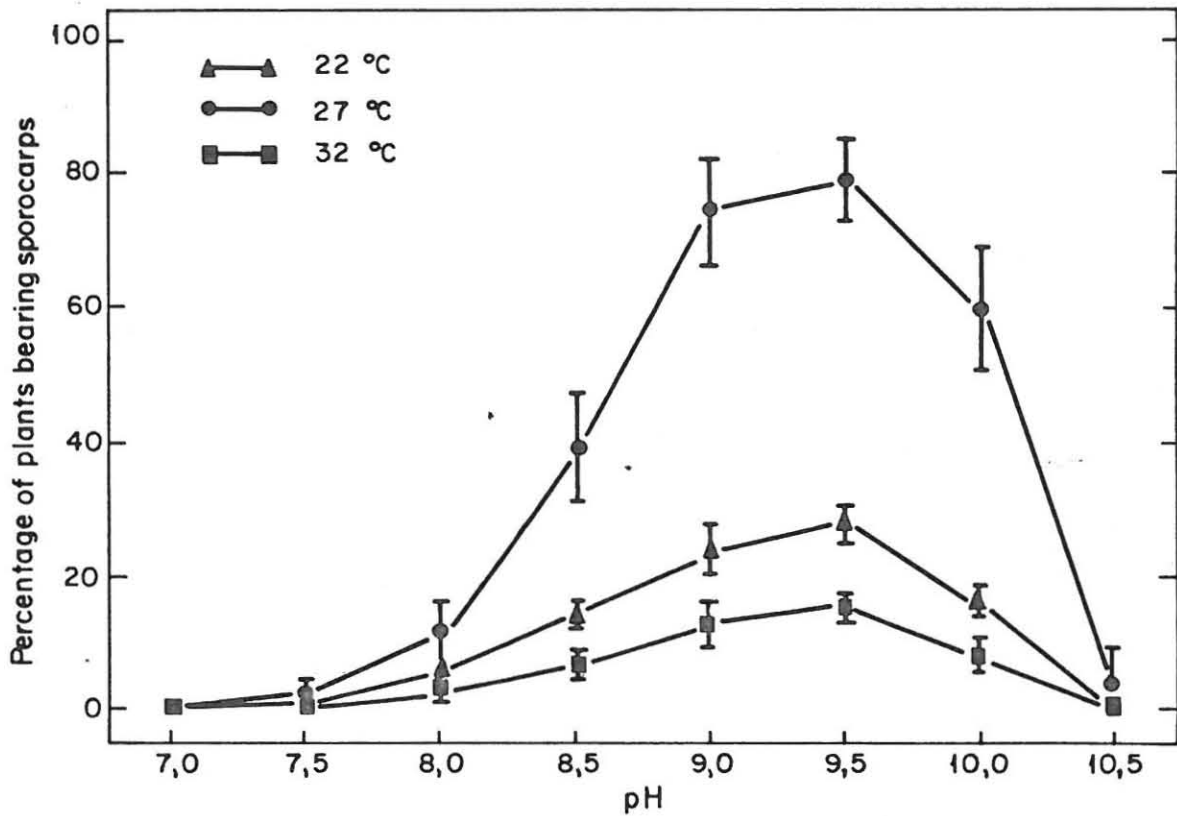


Fig. 16 *The influence of pH and temperature on the number of Azolla filiculoides plants bearing sporocarps. (Vertical bars indicate 95% confidence limits). [30kLux; 14 hour day].*

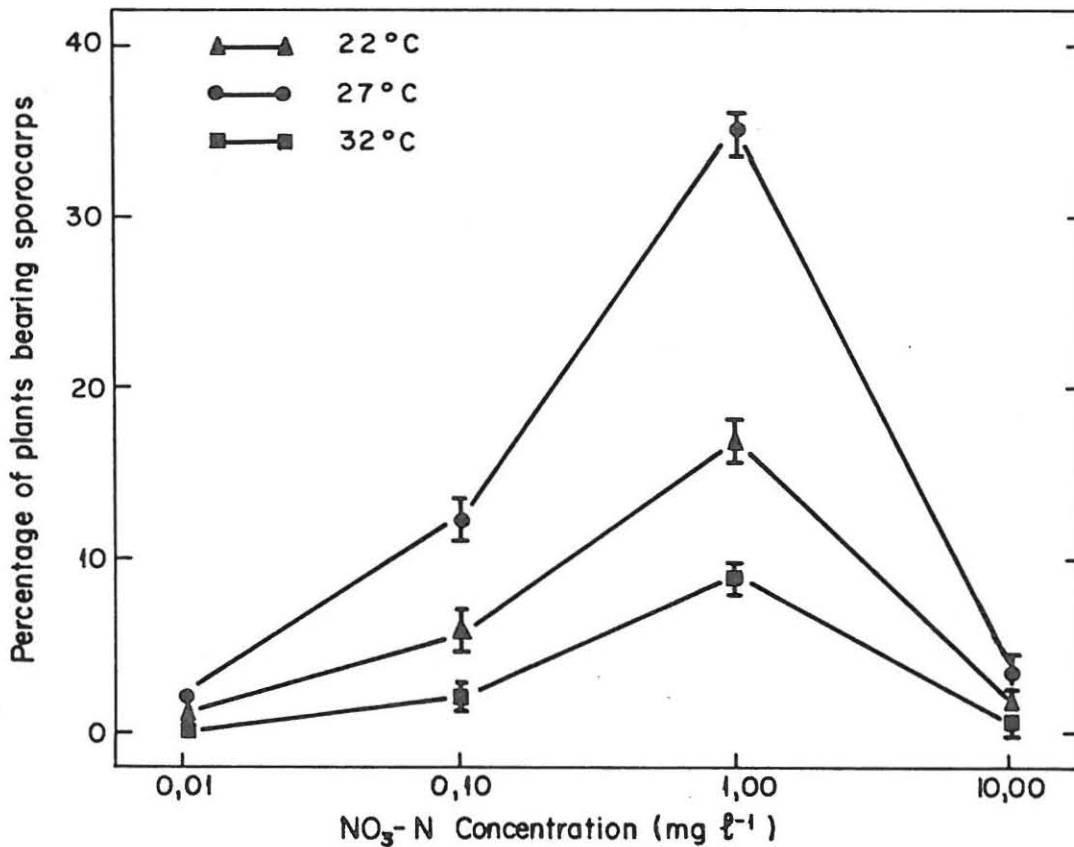


Fig. 17 *The effects of NO<sub>3</sub>-N concentration and temperature on the number of Azolla filiculoides plants bearing sporocarps. (Vertical bars indicate 95% confidence limits). [30k Lux; 14 hour day ; pH 9,5].*

TABLE 3 - The effect of increasing plant density on the mean relative growth rate ( $\overline{\text{RGR}}$ ), ethylene production rate (EPR), doubling time and percentage of *A. filiculoides* plants bearing sporocarps (30 kLux; 14-hour day; 27,5 °C; N-plus medium)

Time elapsed (days)	$\overline{\text{RGR}}$ (%.day <sup>-1</sup> )	EPR (nMoles.(g.f.wt) <sup>-1</sup> .hr <sup>-1</sup> )	Plant density <sub>-2</sub> (No. of plants.dm <sup>-2</sup> )	Doubling time (days)	% of plants with sporocarps
0	20,30	3140	3,18	3,42	0
5	20,30	2890	8,78	3,42	0
10	20,36	2610	24,31	3,39	0
15	16,51	1160	55,49	4,20	0
20	6,72	570	77,64	10,35	0
25	2,89	275	89,73	23,90	2,8
40	0,89	74	104,24	77,02	29,1
60	0,61	41	117,86	115,52	43,4
80	0,32	23	125,75	231,05	47,2

10 mg.ℓ<sup>-1</sup> was inhibitory. Temperature had an effect in that the percentage of plants bearing sporocarps for a given NO<sub>3</sub>-N level were influenced by the temperature; the maximal difference was at 1 mg.ℓ<sup>-1</sup> NO<sub>3</sub>-N. Maximum sporocarp production was observed at 27 °C.

#### 4.4.5.2 Field studies

In many of the streams in the study area, deep pools had been formed by flood-scouring of soft shales and mudstones behind dolerite and limestone ridges which lie at right-angles to the direction of stream flow. After the annual summer flooding, the flow-rate in these streams was reduced to a low but continuous level. The pH of the water varied between 8,2 and 9,4 with high levels of calcium, magnesium, sulphate and carbonate/bicarbonate and very low levels of nitrogen and phosphorus (see Chapter 6). When a mat of *A. filiculoides* developed over a pool, the plants in the lower layers of the mats were often in a state of decomposition and a thick organic layer of sedimented plant material developed on the floor of the pool. Bacterial and chemical degradation of these organic sediments caused the development of anaerobic conditions in the bottom waters and a consequent lowering of the pH of the water under the mats. The levels of nutrients, particularly phosphorus, nitrogen, iron and manganese, were increased due to their liberation from these sediments. The waters immediately below the *Azolla* mats still contained some dissolved oxygen.

The low but continuous inflow of nutrient-poor high pH water immediately below the mat became partly mixed with the nutrient-rich lower water and caused the development of intense pH stratification (Figure 18). From June 1972 to November 1972 the concentrations of PO<sub>4</sub>-P and NO<sub>3</sub>-N, at a depth of 5 cm below the *Azolla* mat at the riverine station, increased from approximately 10 µg ℓ<sup>-1</sup> to 650 µg ℓ<sup>-1</sup> and 35 µg ℓ<sup>-1</sup> to 1 500 µg ℓ<sup>-1</sup> respectively. This partial mixing of the nutrient-poor high pH inflow with the nutrient-rich lower waters ensured that sufficient nutrients, particularly nitrate and phosphate, were available for continued proliferation of the mat and sporocarp development, while the pH of the surface water was maintained at a high level. Although the conditions of pH and nitrate concentration in these environments were thus favourable for sporocarp production from early spring, maximum sporocarp production only

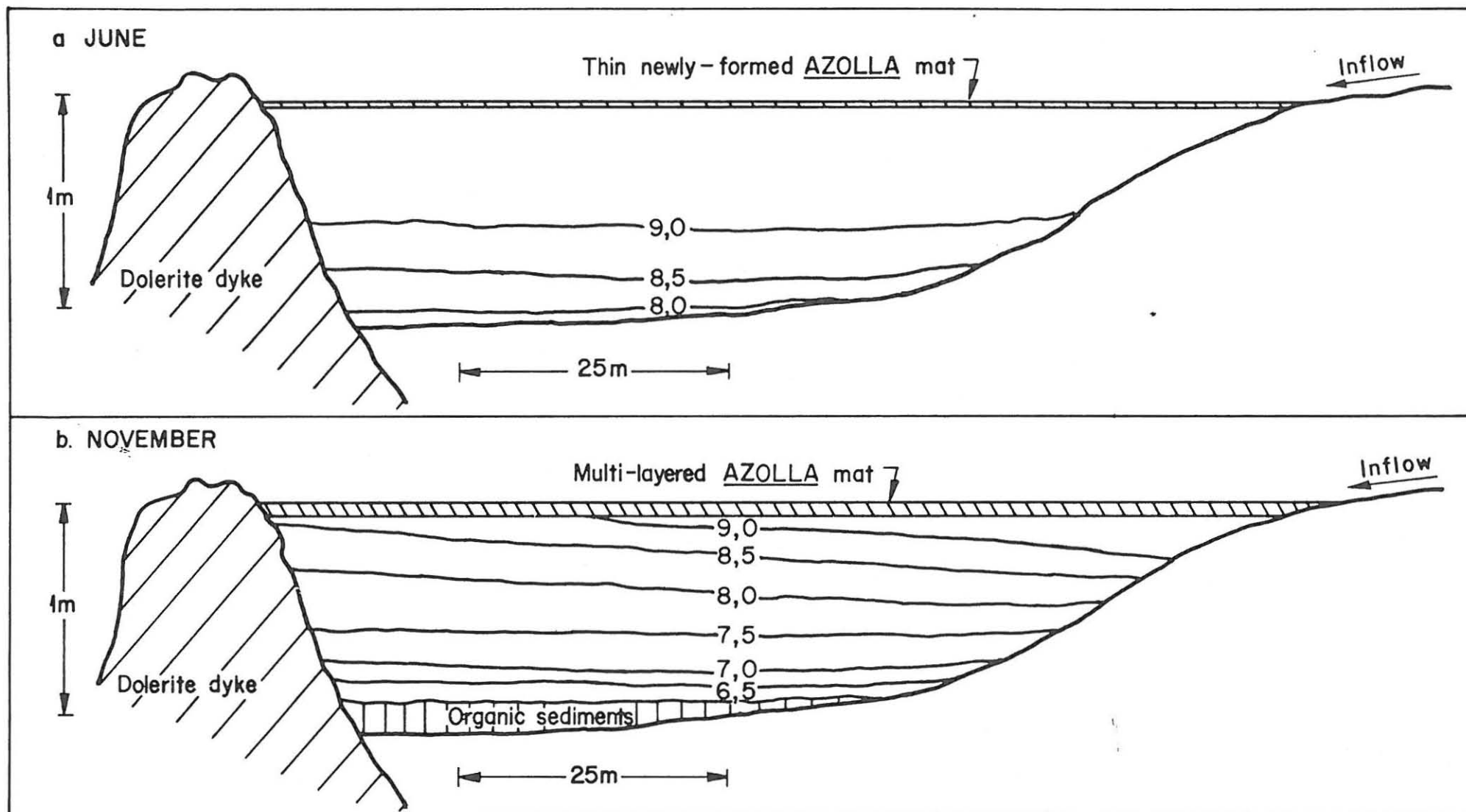


Fig. 18 The development of intense pH stratification beneath a developing *Azolla* mat, from (a) June 1972 to (b) November 1972, at a riverine station receiving a continuous inflow (Horizontal scale greatly reduced) [Station 10].

took place in early summer when the conditions of temperature and photoperiod were more favourable.

In the case of *A. filiculoides* plants growing in a small farm dam which received intermittent inflows, the situation was somewhat different. As in the case of the riverine station, bacterial and chemical degradation of sedimented plant material below the *Azolla* mat caused anaerobic conditions to develop with a decrease in the pH level of the water (Figure 19). The levels of phosphorus, nitrogen, iron and manganese released from these sediments also increased markedly. However, since there was no inflow of high pH water, pH stratification did not develop to the same extent as the riverine station and the pH of the surface waters was therefore lower.

Evapotranspiration from the *Azolla* mat (measured as  $420 \text{ m}^2 \cdot \text{day}^{-1}$  in the laboratory, at 60 kLux and 27 °C) would have caused a lowering of the water level in the field and further increased the concentrations of available nutrients. From June 1972 to November 1972 the concentrations of  $\text{PO}_4\text{-P}$  and  $\text{NO}_3\text{-N}$  at a depth of 5 cm below the *Azolla* mat at the farm dam station increased from approximately  $12 \mu\text{g l}^{-1}$  to  $2\ 900 \mu\text{g l}^{-1}$  and  $30 \mu\text{g l}^{-1}$  to  $6\ 200 \mu\text{g l}^{-1}$  respectively. Despite being approximately four times higher than those recorded in the riverine station, these high nutrient levels caused only a slight increase in the growth of *A. filiculoides*. However, due to the low pH level and high nitrate concentration of the surface water, sporocarp formation was inhibited. It therefore appeared that sporocarp formation would only be initiated when the dam received inflows of nutrient-poor, high pH water following rainfall.

When the monthly values for surface water temperature, pH and percentage of *Azolla* plants bearing sporocarps in the two habitat types were compared with the mean monthly rainfall records for the study period (Figure 20), the differences between the two habitat types were clearly defined. In the riverine station receiving a continuous inflow of high pH water, the pH values remained in the optimum range and maximum sporocarp production was only recorded when the water temperature rose above 20 °C. On the other hand, in the farm dam station which only received inflows after

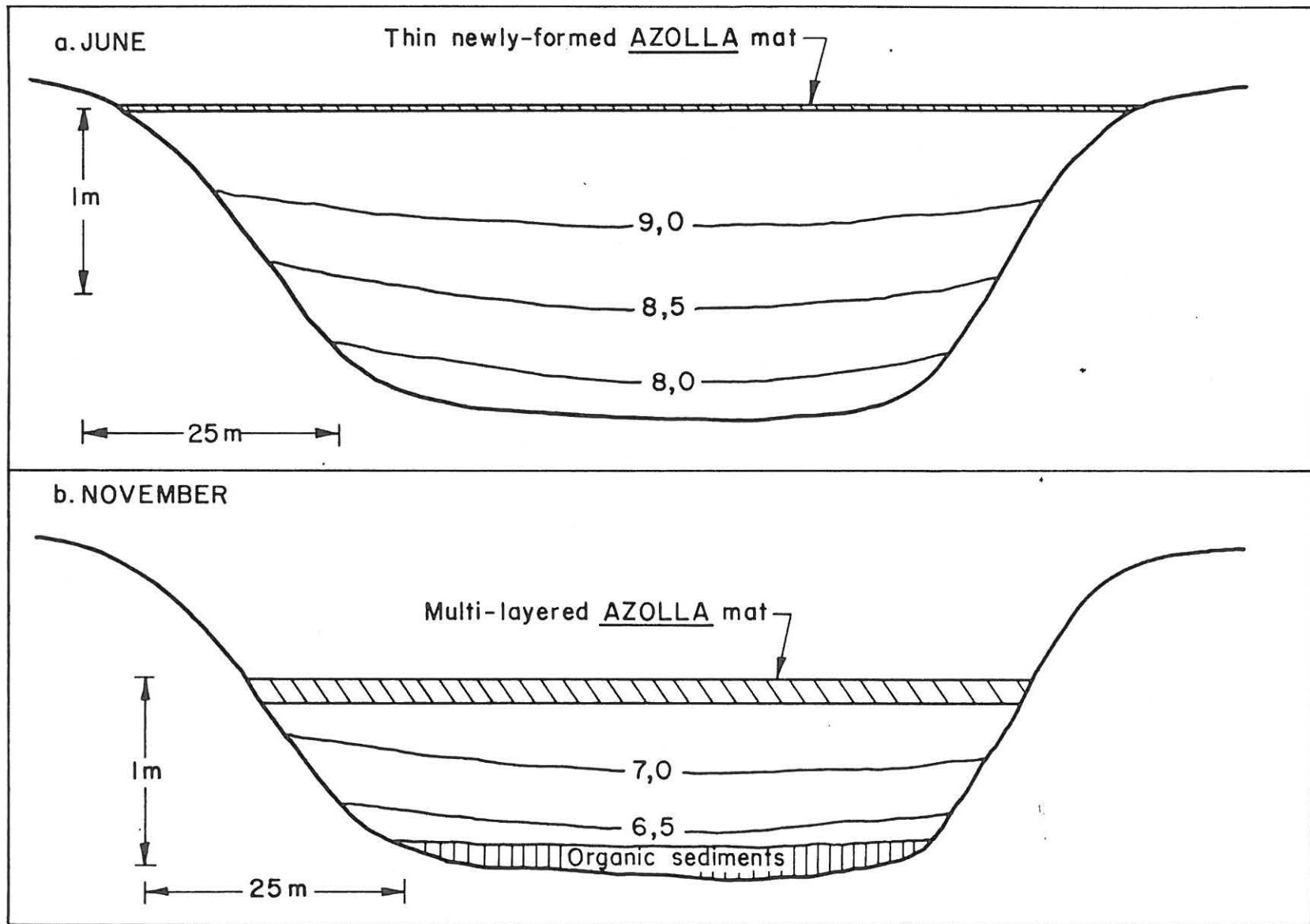
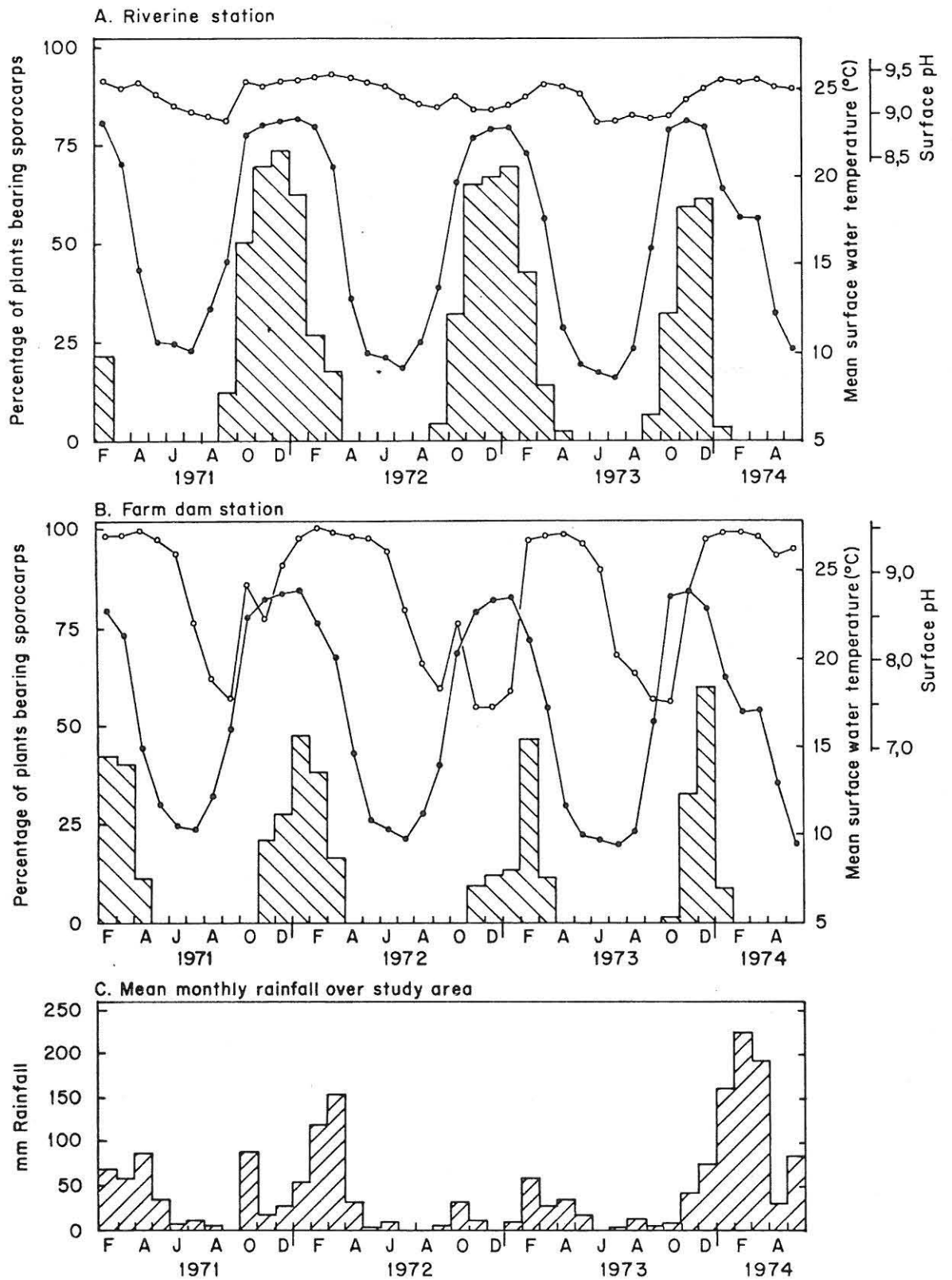


Fig.19 *The decrease in pH caused by anaerobic conditions beneath an Azolla mat developing on a farm dam receiving no inflow during the period (a) June 1972 to (b) November 1972. (Horizontal scale greatly reduced.) [Station 9].*



**Fig. 20** A comparison of the influence of mean surface water temperature (—○—) and pH (—●—) on sporocarp production (histograms) by *Azolla* plants growing at (A) a riverine station with continuous inflow, and (B) a farm dam with intermittent inflow, from February 1971 to May 1974. [Figure C shows the mean monthly rainfall for the study area during this period]

rainfall, the surface pH values decreased to a level unfavourable for sporocarp formation with increasing mat development. Despite the rise in temperature to an optimum level in September, sporocarp formation was dependent on the inflow of high pH runoff following rains in October. This increase in the surface pH value was of short duration, but was enough to trigger off sporocarp formation, despite low rainfalls in November. Rainfall during December and January increased sporocarp formation to a maximum. This was, however, always lower than the riverine station.

In both the riverine and farm dam stations, heavy rainfalls from January to March caused flooding, with a consequent dispersal of the *Azolla* plants and sporocarps. After these annual floods, surviving plants and sporocarps of *A. filiculoides* proliferated to reform the original mat conditions in the streams and dams.

#### 4.4.6 Factors regulating megaspore germination

##### 4.4.6.1 Laboratory studies

At several sampling sites located on streams and farm dams in the study area, large numbers of germinated *Azolla* sporelings were found from December to early April. In some cases it was noted that the habitat had completely dried out at the end of the previous summer, destroying the existing populations of mature *Azolla* plants. Reinfestation of these habitats suggested that megaspores had some degree of resistance to desiccation and low temperatures. In order to investigate these aspects, laboratory experiments were carried out to assess the effects of desiccation, turbulence, photoperiod, light intensity, temperature and pH on the germination of *Azolla* megaspores.

##### (a) Desiccation

The temperature at which megaspores were desiccated had a marked effect on megaspore germination (Figure 21). In addition, desiccation for a period longer than 40 days greatly reduced megaspore germination at the three temperatures used. The percentage of germinating megaspores decreased very rapidly with increasing periods of desiccation at 35 °C, compared with the germination after similar periods of desiccation at both 5 °C and

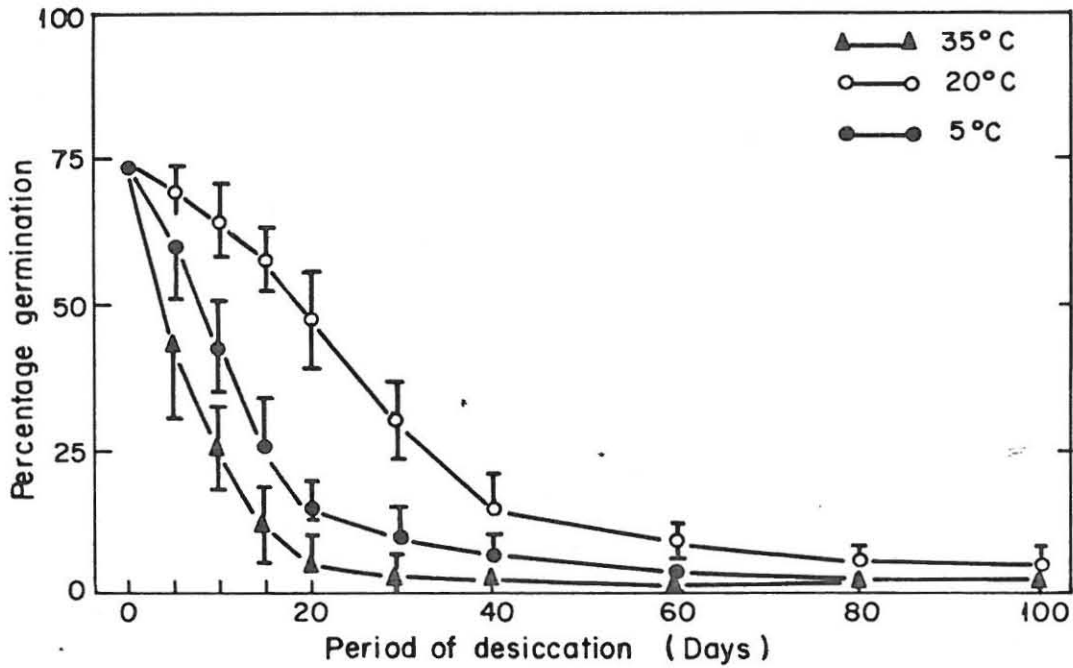


Fig. 21 *The effects of desiccation at three different temperatures on the germination of Azolla filiculoides megaspores. (Vertical bars indicate two standard errors). [30kLux; 14 hour day; pH 7,0].*

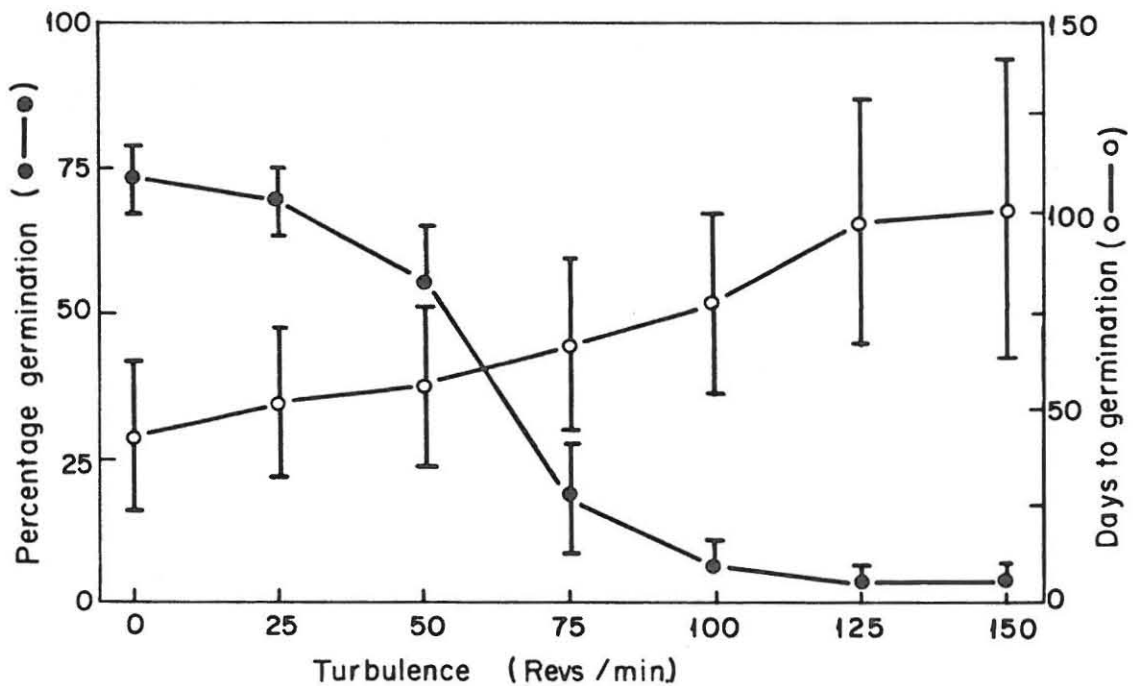


Fig. 22 *The effects of increasing turbulence on the germination of Azolla filiculoides megaspores. (Vertical bars indicate two standard errors). [30 kLux; pH 7,0; 20°C; 14 hour day].*

20 °C. Maximum germination (75 %) was recorded with no desiccation whilst a period of 100 days desiccation at all three temperatures reduced germination to 5 %.

(b) Turbulence

To test the effects of turbulence on megaspore germination, megaspores were placed in a 300 ml Erlenmeyer flask containing 100 ml of nutrient solution (Table 2) and shaken on a Bühler rotary shaker with an orbit radius of 3 cm. Each experimental treatment consisted of six replicate flasks containing 50 megaspores, and each experiment was repeated three times. A direct relationship cannot be made between stream current speed or wave action and the turbulence generated in the experimental apparatus. However, a comparison can be drawn between the degree of turbulence, i.e. whether mild, moderate or severe. The results are shown in Figure 22.

Mild turbulence (0-50 revolutions per minute) caused a decrease in megaspore germination from 74 % to 57 %, whereas moderate turbulence (50-90 revolutions per minute) further decreased germination to approximately 10 %. Severe turbulence (>100 revolutions per minute) reduced megaspore germination to a low, but nearly constant, level of approximately 6 %. In this series of experiments, the germination time (i.e. the length of time from initiation of the experiment to germination) increased approximately three-fold when the turbulence was increased from 0 to 150 revolutions per minute.

(c) Photoperiod

Photoperiod and water temperature were found to have interacting effects on the germination of *Azolla* megaspores (Figure 23). At a temperature of 10 °C, megaspore germination only occurred at photoperiods of 10 hours or greater, and then only at a very low level (maximum 6 %). At temperatures of 20 °C and 30 °C, germination was initiated at a photoperiod of 9 hours, rising rapidly to a peak at 14 hours. Further increases in photoperiod caused slight increases in the percentage germination at both 20 °C and 30 °C. The percentage germination at 30 °C was lower than that at 20 °C for all the photoperiods used.

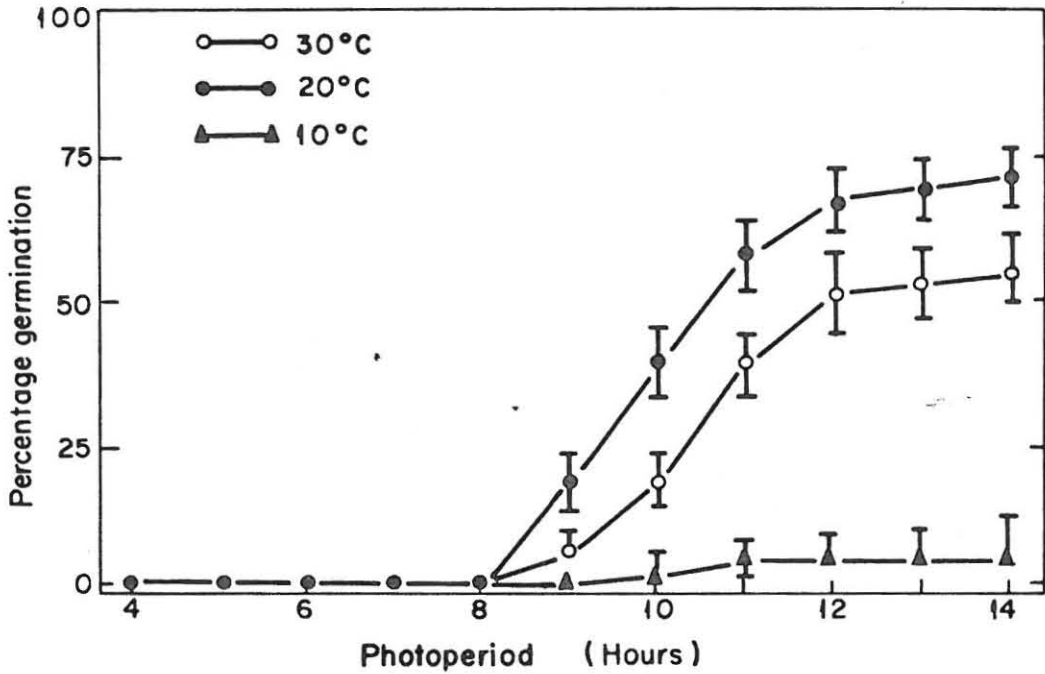


Fig. 23 *The interacting effects of water temperature and photoperiod on the germination of Azolla filiculoides megaspores. (Vertical bars indicate two standard errors). [30kLux; pH 7,0].*

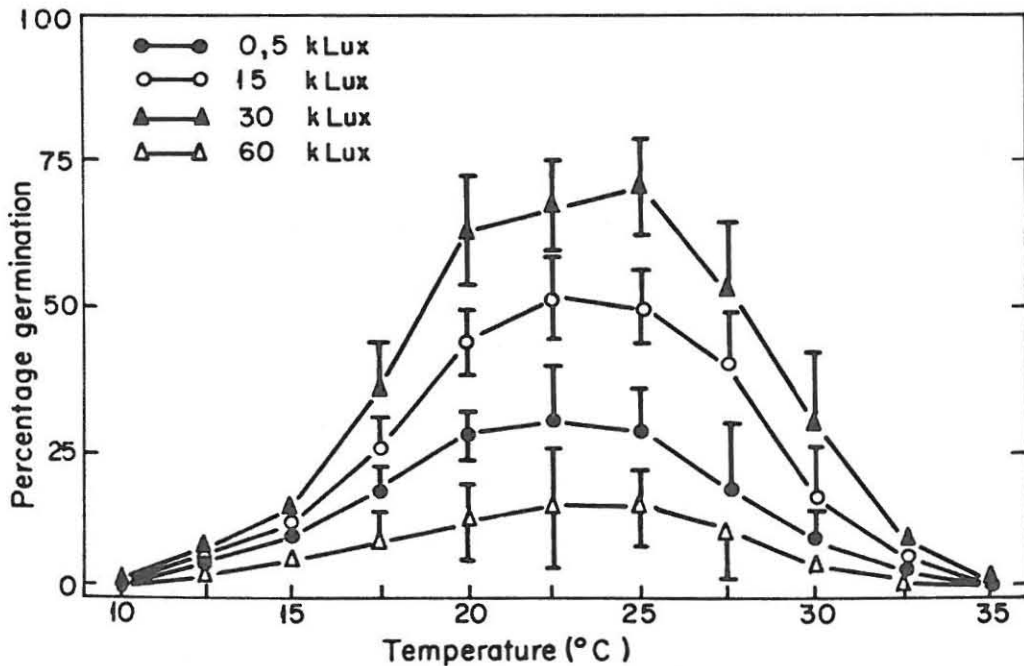


Fig. 24 *The interacting effects of light intensity and water temperature on the germination of Azolla filiculoides megaspores. (Vertical bars indicate two standard errors). [pH 7,0; 14 hour day].*

(d) Light intensity

Light intensity and temperature were also found to have interacting effects on the germination of *Azolla* megaspores (Figure 24). With increasing light intensity from 0,5 kLux to 30 kLux, megaspore germination increased over a wide temperature range (12,5 °C to 32,5 °C). No germination was recorded at 35 °C and only a very low germination rate (0,7 %) was recorded at 10 °C. At 60 kLux megaspore germination decreased to a minimum, below that recorded at 0,5 kLux. At both 0,5 kLux and 15 kLux, maximum germination occurred at 22,5 °C. However, at both 30 kLux and 60 kLux, maximum germination was recorded at 25 °C.

(e) pH

In common with the previous series of experiments, the pH of the nutrient solution and water temperature had interacting effects on megaspore germination (Figure 25). Germination was observed to take place over a wide pH range (5,0 to 9,0). No germination occurred at pH levels below 4,5 or above 9,5. Maximum megaspore germination was recorded at pH 7,0 for each of the temperatures used (10 °C, 20 °C and 30 °C). The percentage germination was similar at pH 6,0 and 8,0, but slightly lower than that at pH 7,0. The greatest germination occurred at 20 °C, and the least at 10 °C, over the pH range 5,0 to 9,0. At a water temperature of 30 °C, the percentage germination decreased well below the level recorded at 20 °C, but still significantly ( $p < 0,05$ ) greater than at 10 °C.

4.4.6.2 Field studies

To relate the results of laboratory experiments with field observations, monthly data for bottom water pH and temperature were compared with estimates of the relative abundance of *Azolla* sporelings at station 10 (Figure 26).

Measurements and observations made in the field supported the results of laboratory experiments. Newly-germinated *Azolla* sporelings were first found during the mid-summer months when daylengths were long and bottom water temperatures high. During this period the pH of the bottom waters remained low (6,1 to 6,5) except after inflows of high pH water following rain storms, when the pH rose as high as 8,4. These high values decreased

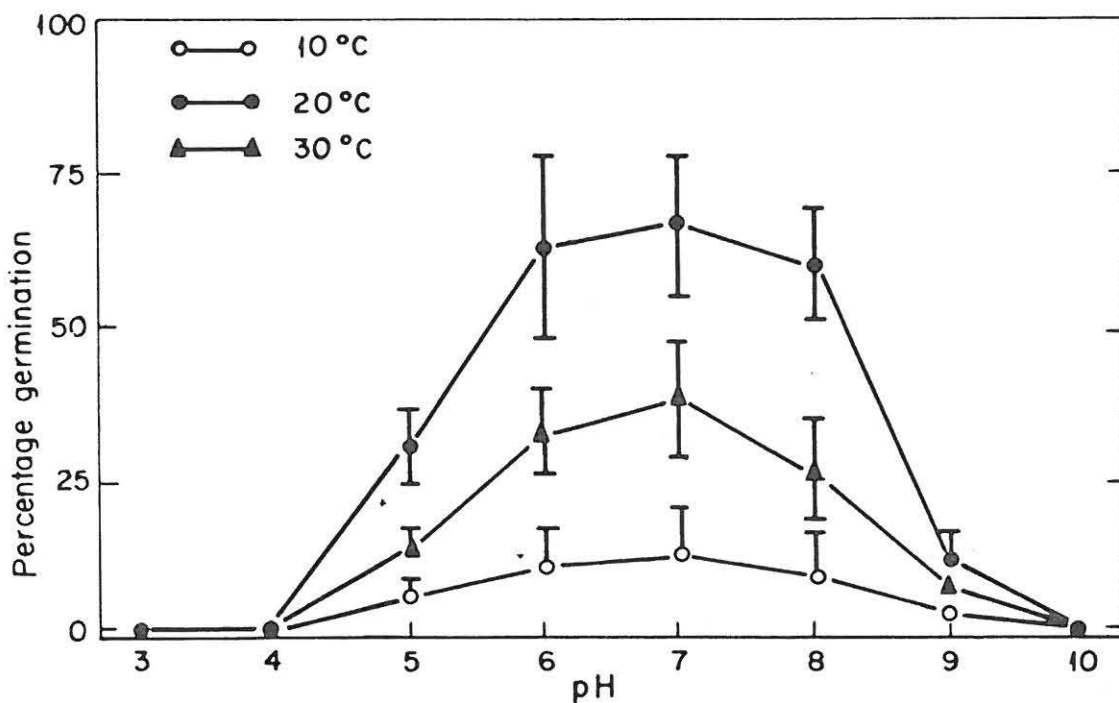


Fig. 25 *The interacting effects of water temperature and pH on the germination of Azolla filiculoides megaspores. (Vertical bars indicate two standard errors). [30 k Lux; 14 hour day].*

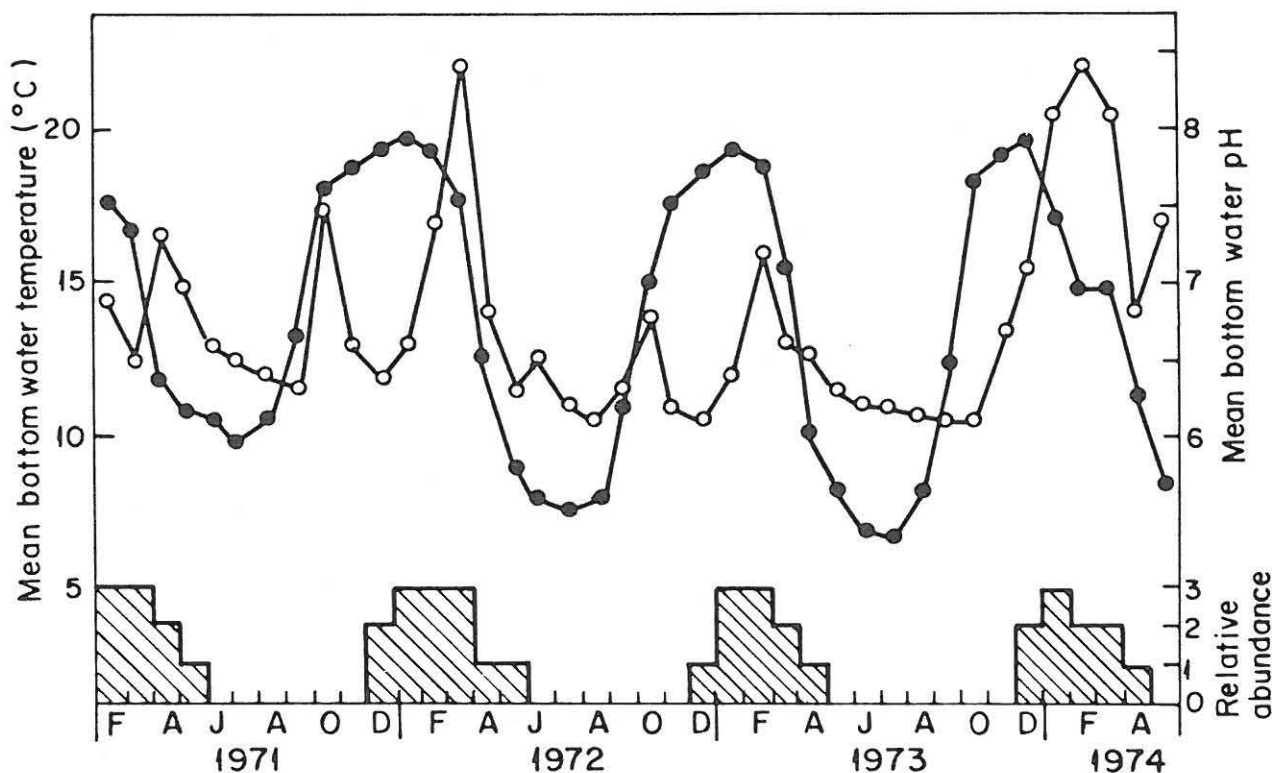


Fig. 26 *A comparison of mean bottom water pH (o—o) and temperature (●—●) with the germination of Azolla filiculoides megaspores (histogram) at a riverine station receiving continuous inflow, from February 1971 to May 1974. [Relative abundance scale : 0 = absent; 1 = scarce; 2 = frequent; 3 = abundant]. (Station 10).*

rapidly with the re-establishment of stratification beneath the *Azolla* mat. No germinated sporelings were found during the periods when a thick, established mat of *Azolla* plants covered the water surface. It appears, therefore, that germination was inhibited in the dark, or if some megaspores did germinate and float towards the surface, the sporelings became trapped in the lower decomposing layers of the *Azolla* mat and perished. When winds blew for prolonged periods, the mat of *Azolla* plants tended to accumulate on the leeward shore of the pool. This permitted light penetration through the water column and stimulated megaspore germination. Similarly, low intensity flooding often washed out large portions of the *Azolla* mat at certain of the sampling sites, without scouring the pool and removing the sedimented megaspores. This again permitted light penetration and stimulated megaspore germination. The ability of megaspores to tolerate low water temperatures is reflected in the appearance of newly-germinated sporelings during the autumn months when water temperatures had dropped to approximately 9 °C.

#### 4.4.7 Population fluctuations of *Azolla filiculoides* in the study area

The population fluctuations of *A. filiculoides* at each of the twelve sampling sites in the study area are shown in Figures 27 to 35. Because the sites differed greatly from each other in size and hydrology (Table 1), areal density was chosen as the most suitable method of comparing the plant populations.

The pattern of plant density fluctuations was generally similar at each of the twelve sampling sites. At the riverine sampling sites (stations 1-7, 10 and 11), plant densities were lowest during the late summer months, January to April, as a result of the flushing effects of the annual floods. After the water levels had receded, plant densities increased through winter to a mid-summer maximum during November and December. This increase was due both to the rapid vegetative reproduction of surviving plants and the germination of sporocarps. However, sampling sites 1, 2 and 3 were ruined by the dredging of their respective streams during July 1972 for the purpose of constructing new bridges along the Bethulie-Oranje-krag road (Figure 4). This dredging completely altered the hydrological regime of the streams concerned and resulted in the disappearance of *Azolla* plants from these sites (Figures 27, 28 and 29).

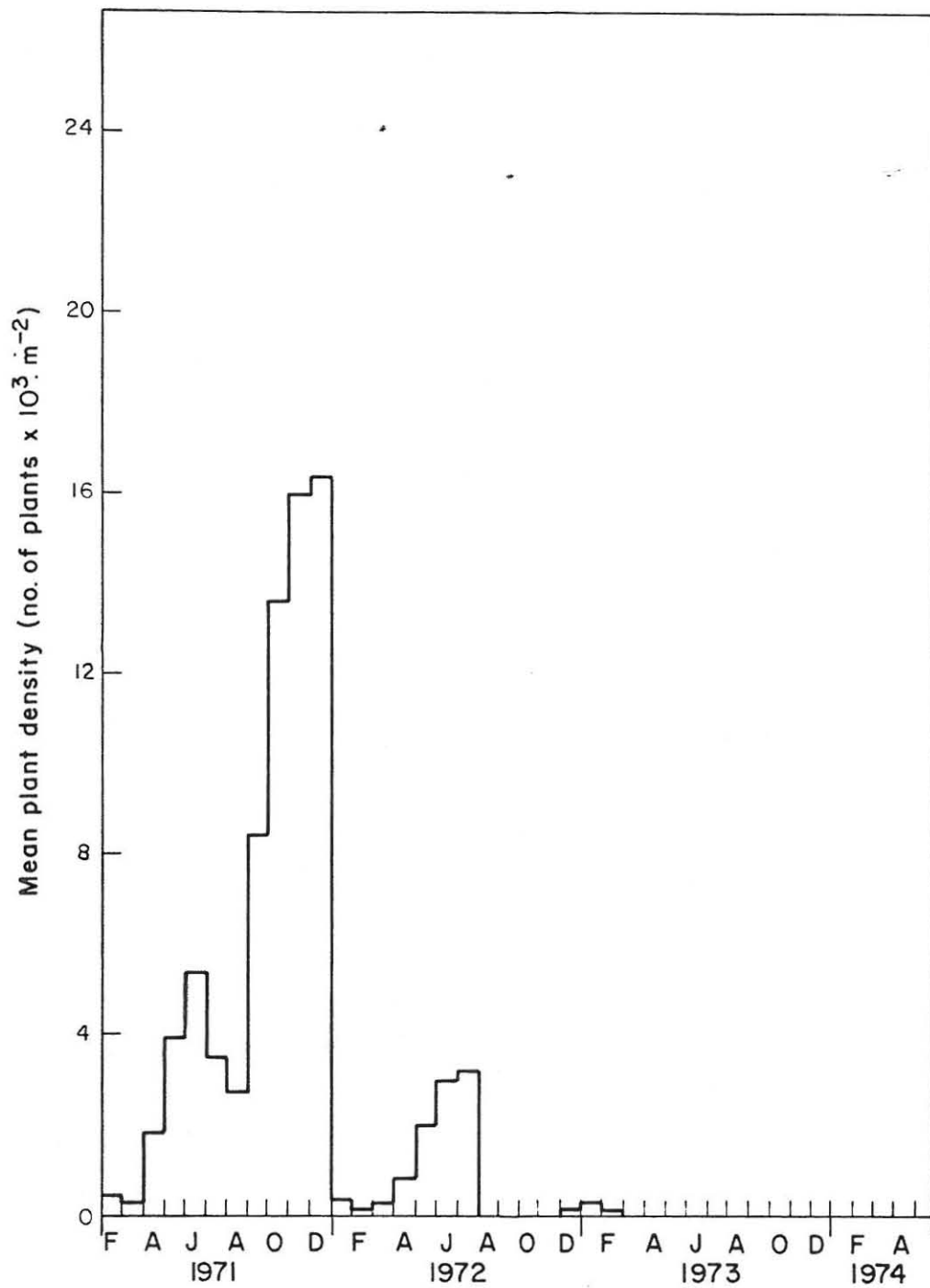


Fig. 27 *Histogram of mean plant density at station 1, from February 1971 to May 1974.*

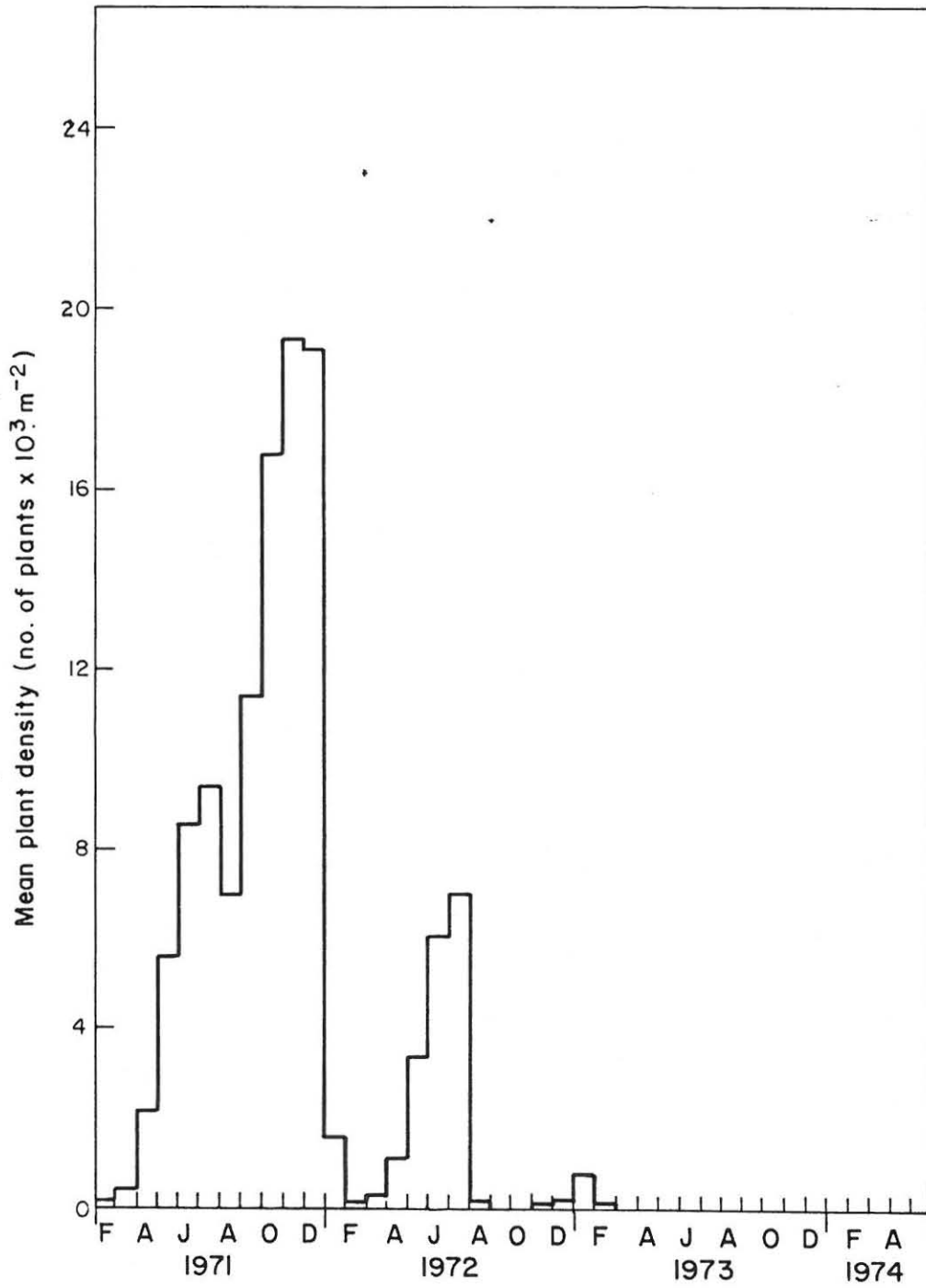


Fig. 28 *Histogram of mean plant density at station 2, from February 1971 to May 1974.*

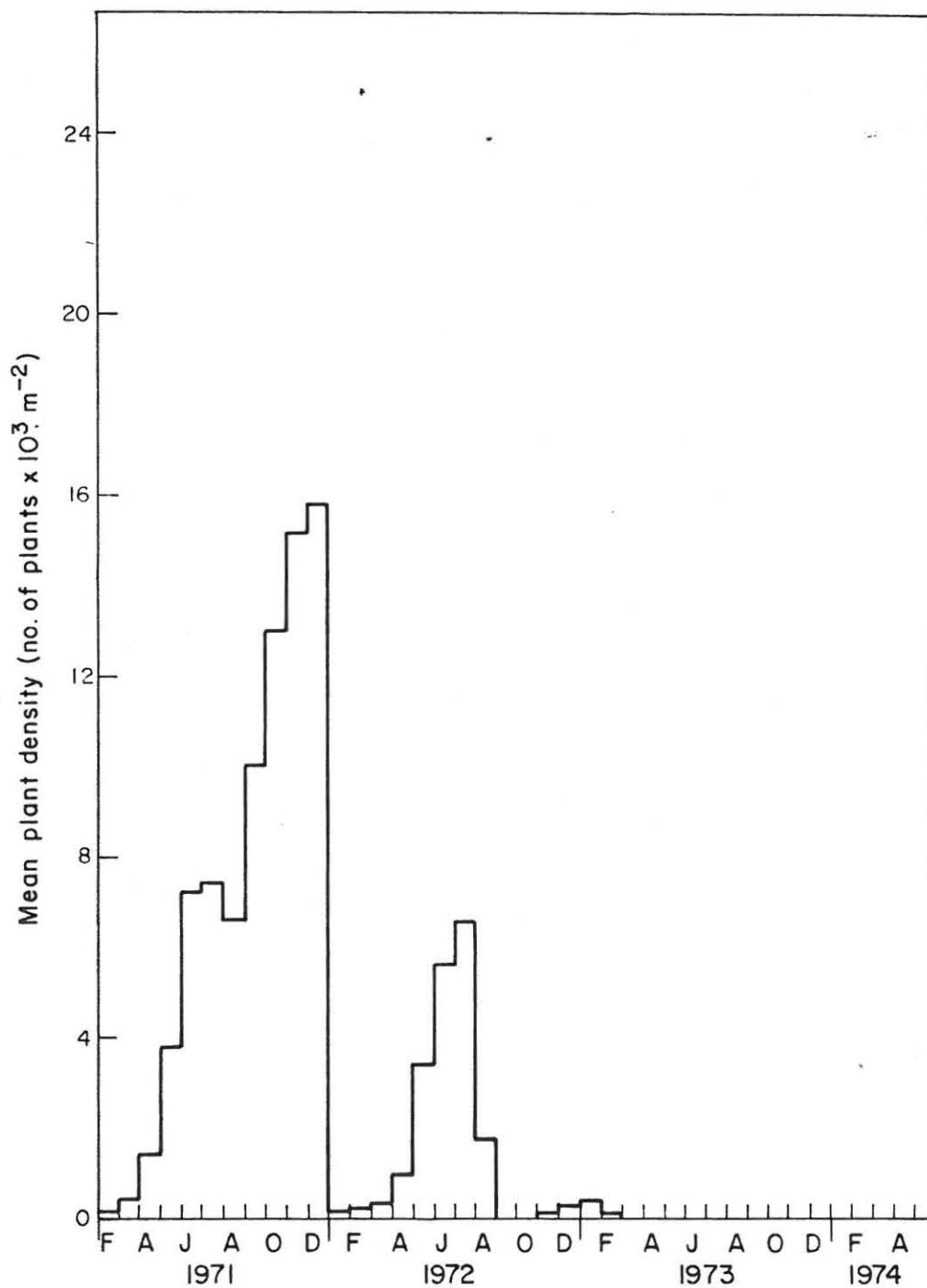


Fig. 29 Histogram of mean plant density at station 3, from February 1971 to May 1974.

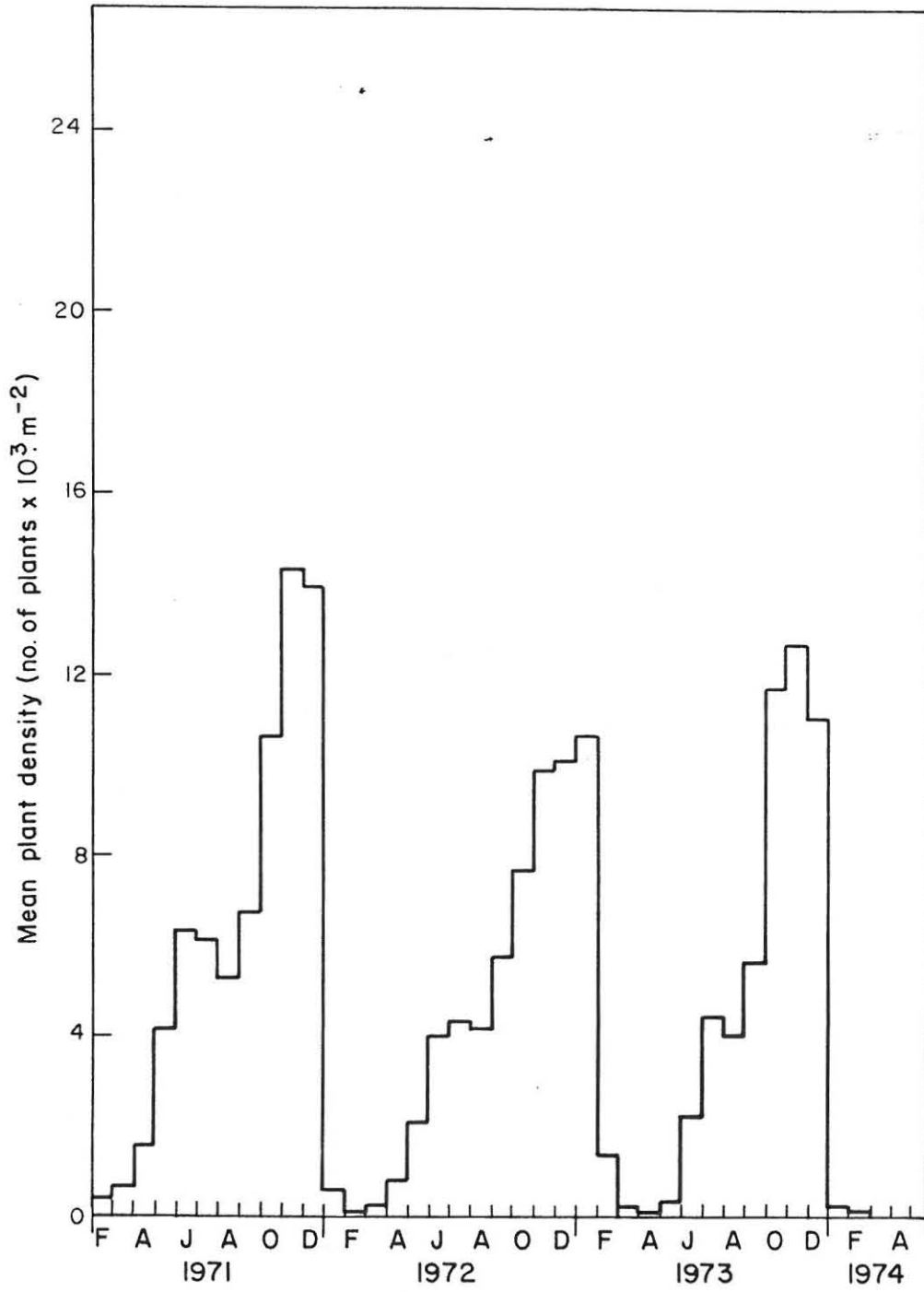


Fig. 30 *Histogram of mean plant density at station 4, from February 1971 to May 1974.*

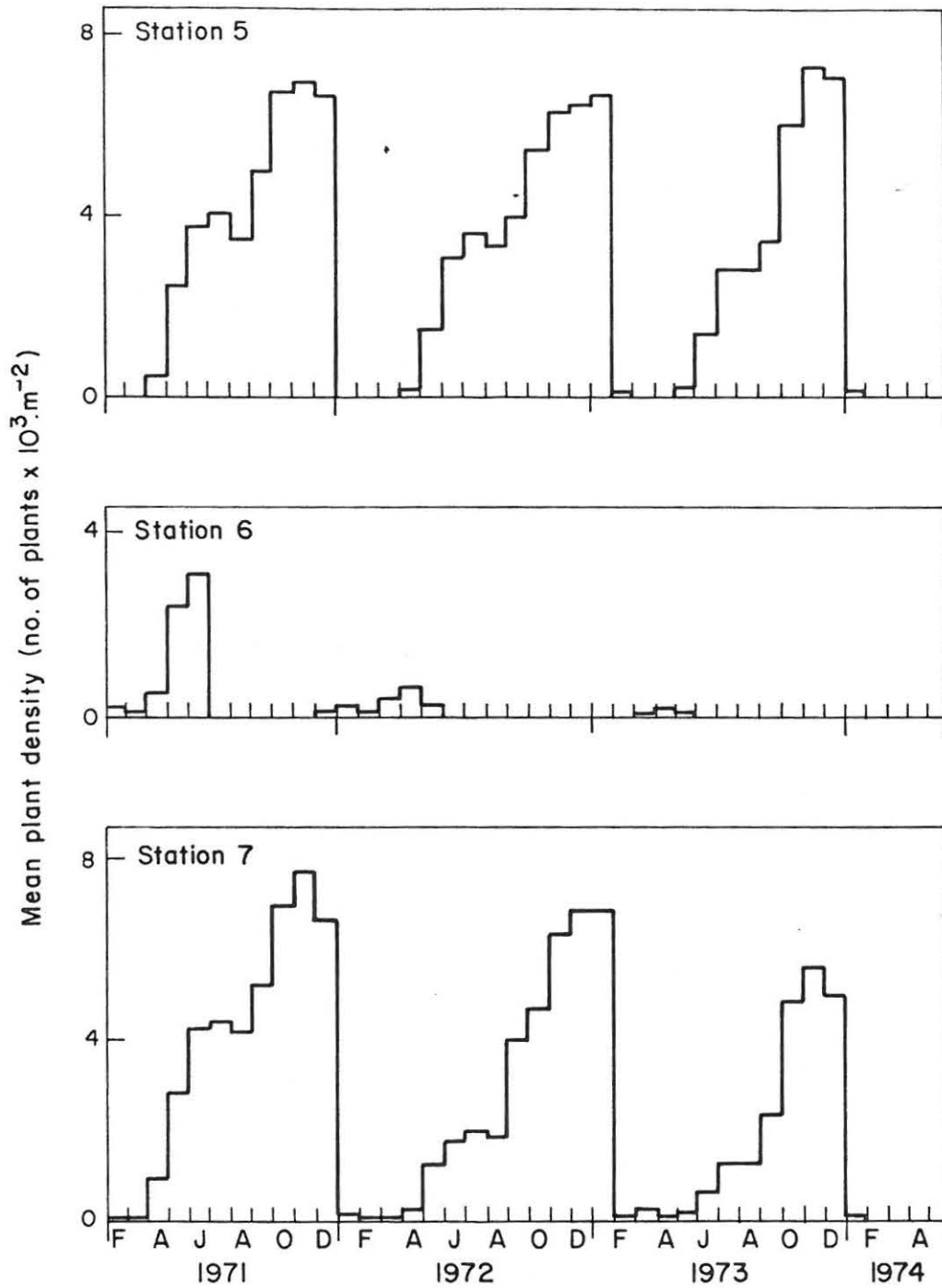


Fig. 31 *Histograms of mean plant density at stations 5, 6 & 7, from February 1971 to May 1974.*

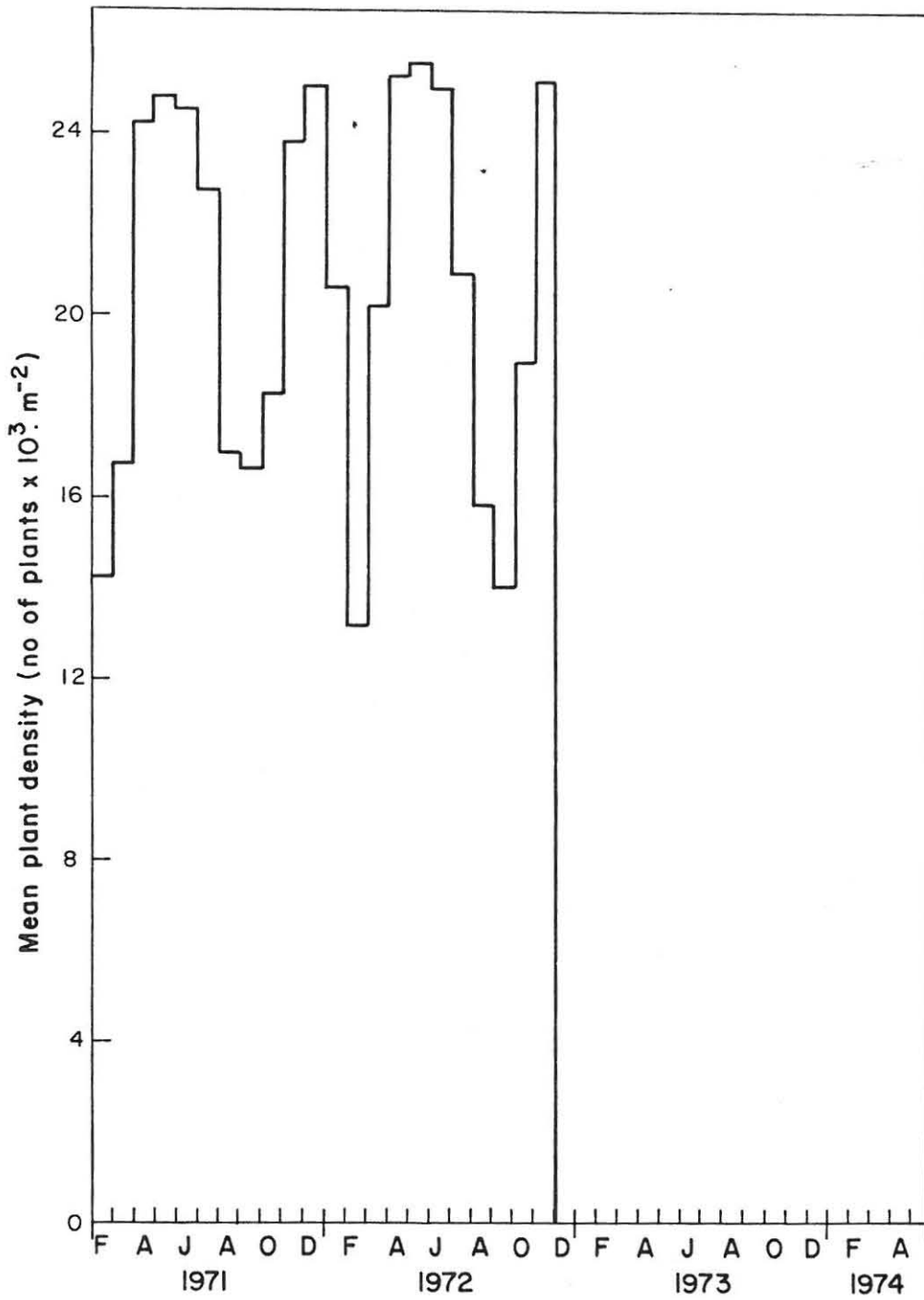


Fig. 32 *Histogram of mean plant density at station 8, from February 1971 to May 1974.*

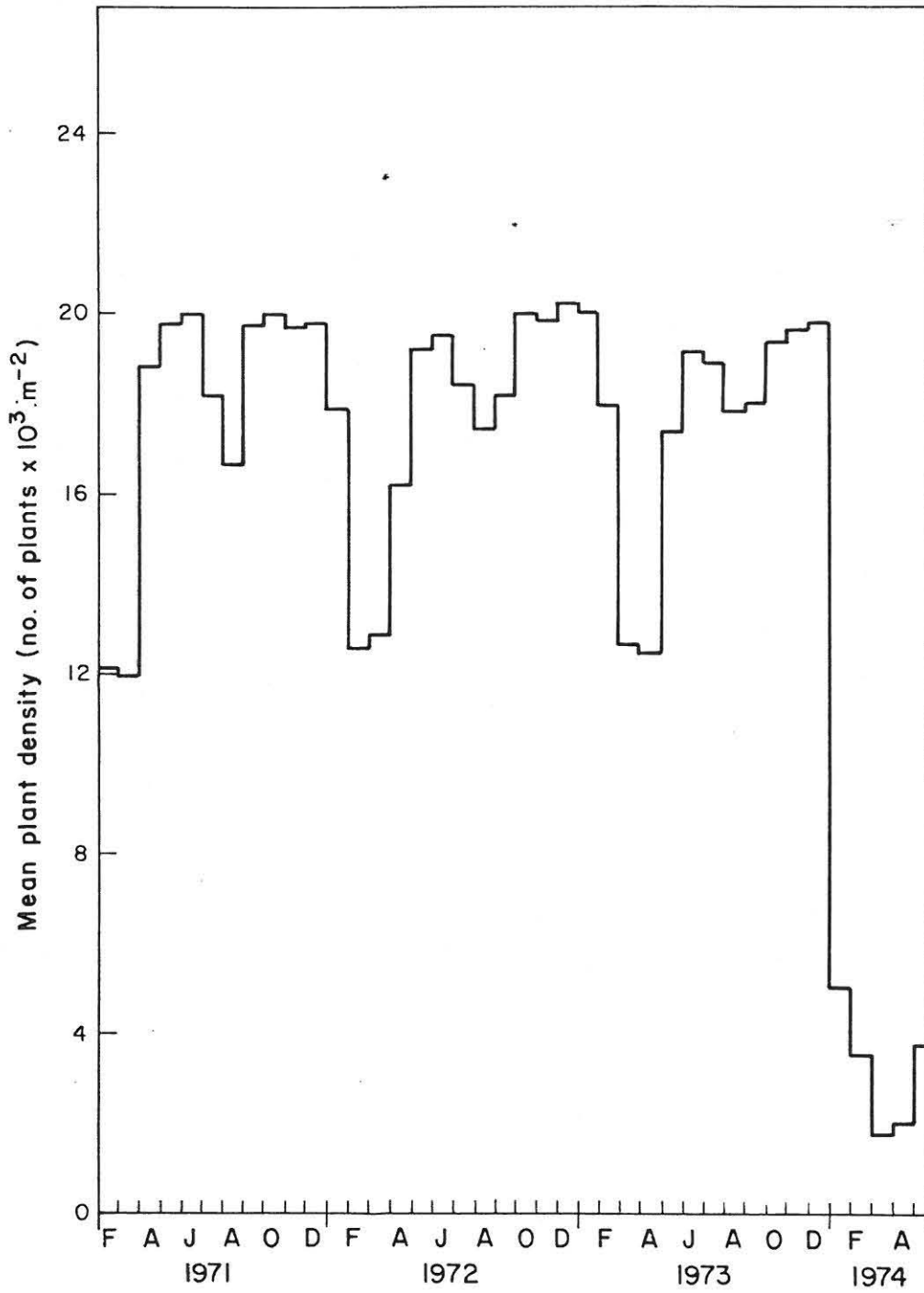


Fig. 33 *Histogram of mean plant density at station 9, from February 1971 to May 1974.*

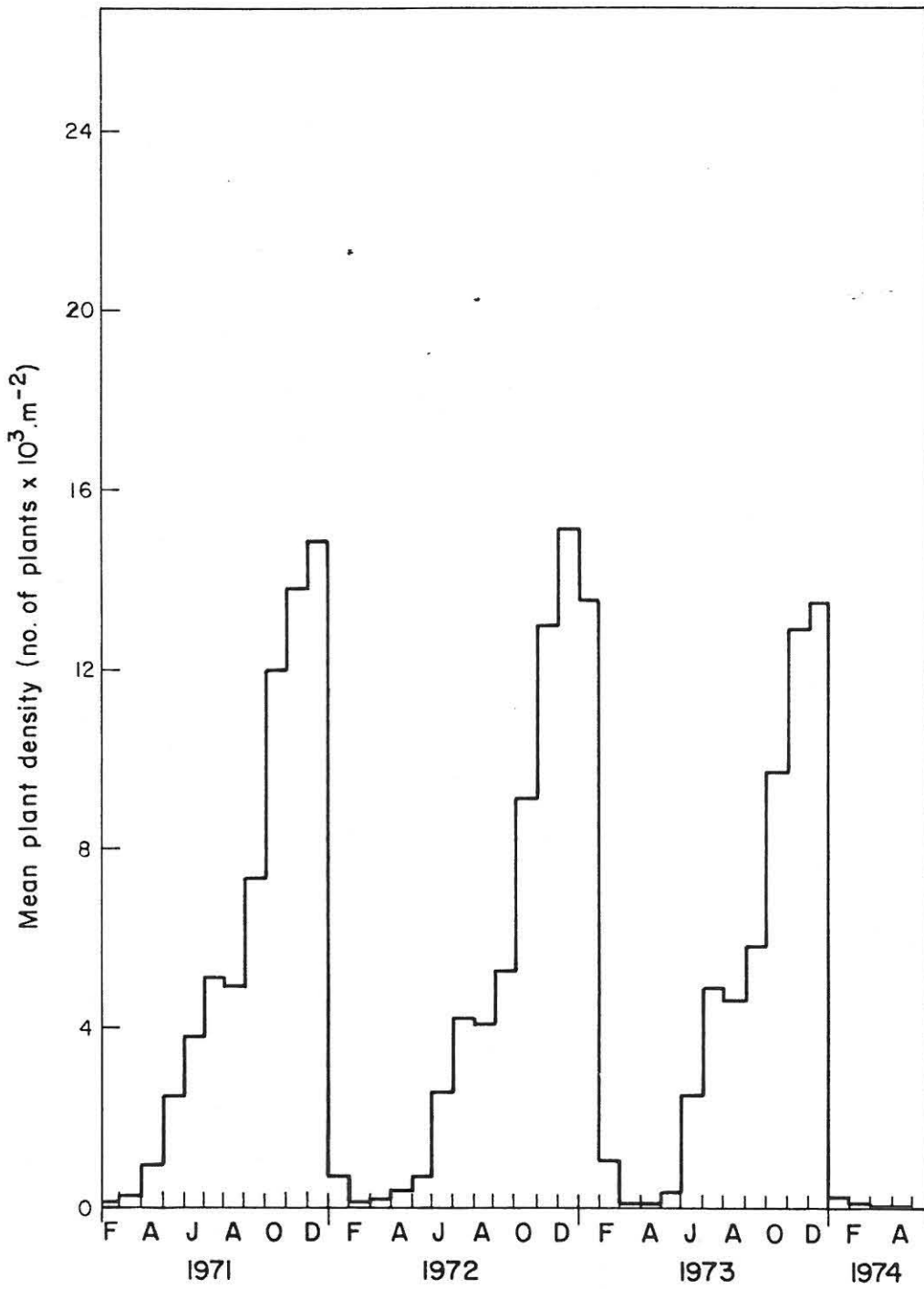


Fig. 34 *Histogram of mean plant density at station 10, from February 1971 to May 1974.*

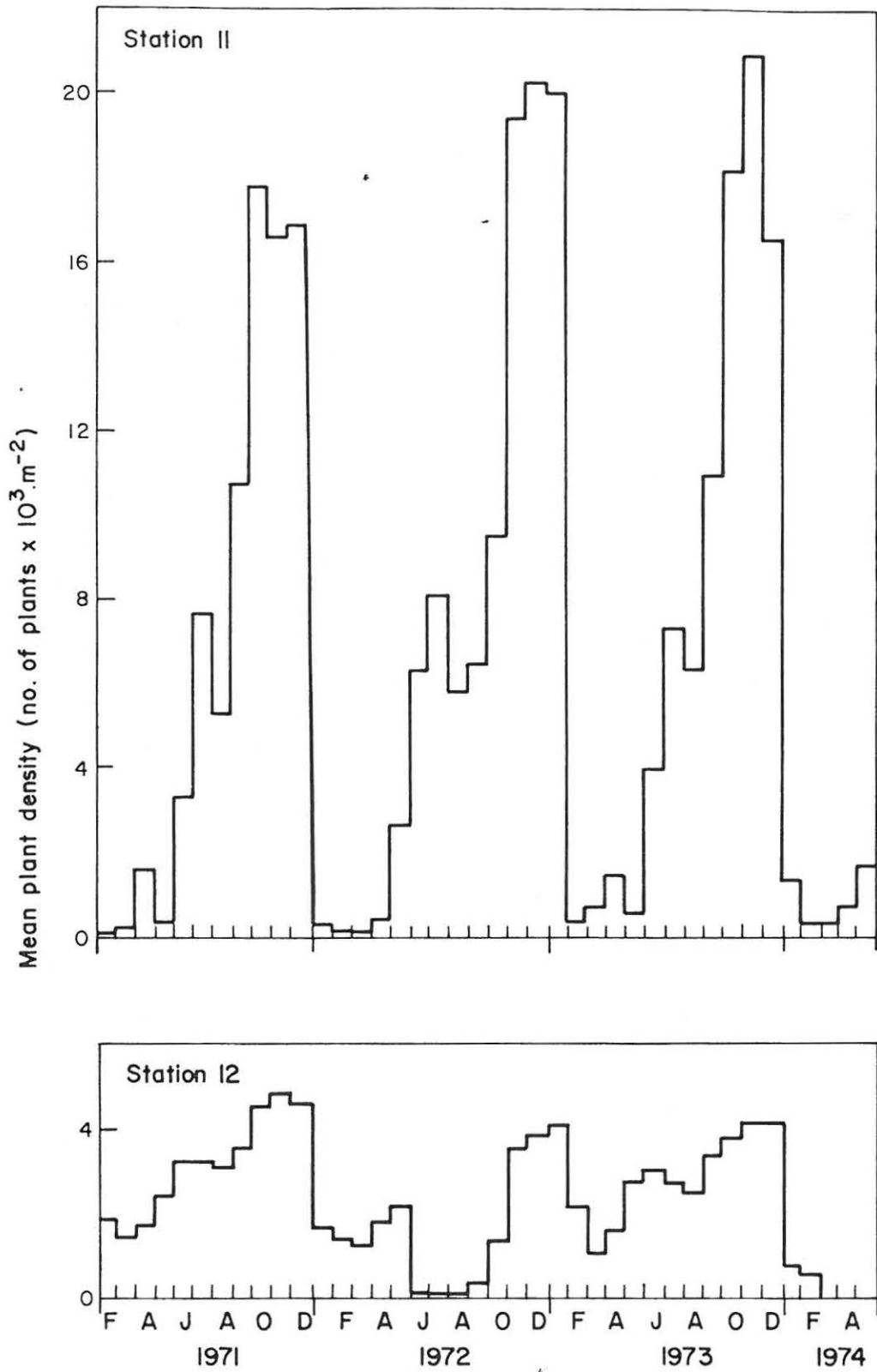


Fig. 35 *Histograms of mean plant density at stations II & 12, from February 1971 to May 1974.*

At the two sampling sites located in small man-made reservoirs (stations 8 and 9; Figures 32 and 33), minimum plant densities coincided with rainfall maxima (Figures 5 and 6), due to spill-over of the plants as water levels rose. At station 9 the very heavy rainfalls recorded during January and March 1974 caused a marked drop in plant density (Figure 33). At station 8, further fluctuations in plant density were caused by the periodic removal of plants from the reservoir by farm workers. In November 1972 this reservoir was cleaned out by the farmer and *A. filiculoides* plants did not reappear (Figure 32). The density of plants growing at station 12, located at the eye of a spring in a dolerite ridge, fluctuated with changes in the delivery rate of the spring (Figure 35). Here again, maximum plant densities were recorded in mid-summer.

At several of the sampling sites, plant densities reached very high levels, e.g. 25 000 m<sup>-2</sup> at station 8 and 20 000 m<sup>-2</sup> at stations 9 and 11. At these sampling sites, the restricted flow of water prevented wash-out of the plants until heavy rainfall caused a rapid rise in water level. At station 6, located on a backwater of the Brakspruit, the stream dried out and *Azolla* plants disappeared during the winter of 1971 (Figure 31). The reappearance of small numbers of plants in shallow temporary pools during 1972 and 1973 was probably due to the germination of sporocarps in the mud. However, these plants soon died when the pools dried up.

#### 4.5 DISCUSSION

The morphology and anatomy of *A. filiculoides* plants from the Hendrik Verwoerd Dam catchment area closely matched published descriptions for this species from the northern hemisphere (Campbell, 1893; Queva, 1910; Duncan, 1940; Bonnet, 1957). In the study area, *A. filiculoides* can be distinguished by its size (up to 40 mm in length), the curled appearance of the fronds and the conspicuous (3 to 4 cells wide) hyaline margin to the dorsal leaf lobe. The presence of unicellular papillae on the upper surface of the dorsal leaf lobe, two guard cells per stoma and the virtual absence of root hairs are also characteristic features of *A. filiculoides*. The bright red colour of plants growing in full sunlight is due to the anthocyanins luteolinidin and apigenidin (Pieterse *et al.*, 1977). Shade-grown plants are a translucent greenish-yellow, a darker green central spot on the dorsal leaf lobe marking the presence of

symbiotic algal colonies within the leaf. All the South African material of *A. filiculoides* examined in this study contained these colonies of *A. azollae* within dorsal leaf lobe cavities. In contrast to the observations of Marsh (1914), Huneke (1933) and Hill (1977), who recorded instances of naturally-occurring alga-free plants, no specimens of alga-free *Azolla* plants have been found in South Africa.

Extensive aerenchymatous tissue in the base of both dorsal and ventral leaf lobes provides buoyancy, and *Azolla* plants float with the lower leaf lobe in contact with the water only on its lower surface. Reports that the lower leaf lobe is submerged (Sadebeck, 1902; Huneke, 1933; Smith, 1938; Saubert, 1949) were disproved in this study.

The pattern of development recorded in this investigation, whereby cells of *A. azollae* at the apical meristem are trapped within depressions in the dorsal leaf lobe primordia by epidermal cell growth, is in complete agreement with earlier reports (Sadebeck, 1902; Rao, 1936; Shen, 1960; Konar and Kapoor, 1972). Further leaf development results in the formation of a cavity which is open to the atmosphere via a small pore. The report by Hill (1977) that this *Anabaena*-containing cavity is 'sealed off' during further development is erroneous. Morphological and anatomical descriptions of the *Anabaena*-containing cavities in *Azolla* leaves by Grilli (1964), Konar and Kapoor (1972), Duckett *et al.* (1975a, b), Peters (1976) and Peters, Toia, Raveed and Levine (1978) are supported by the observations made during this investigation.

Within the leaf cavity of *A. filiculoides*, hair cells arise from the epidermal cells lining the cavity. In young leaves these hairs are unicellular, but each hair becomes multicellular as the leaf ages. Filaments of *A. azollae* are arranged around the periphery of the cavity and often adhere to the protruding hair cells. The strategic location of these hair cells in the *Anabaena*-containing leaf cavities of *Azolla* strongly suggests that these cells have an important role in the symbiosis. Each hair cell has dense cytoplasmic contents and, under the electron microscope, conspicuous wall ingrowths, considered by Duckett *et al.* (1975b) to be typical features of transfer cell morphology, are

visible. While numerous examples of transfer cells are known from angiosperms (Gunning and Pate, 1969), far fewer occurrences have been recorded from bryophytes and pteridophytes (Duckett and Prasad, 1974). Perhaps the best-known are the wall labyrinths at gametophyte-sporophyte junctions (Maier and Maier, 1972; Pate and Gunning, 1972). Since this transfer cell morphology has been implicated in the short distance transport of solutes in other anatomical situations, it is highly likely that the same is true in *Azolla*. The increase in the surface area of the cell membrane, brought about by the development of wall ingrowths, is an obvious adaptation facilitating such transport processes.

Although the most likely function of the leaf hair cells is absorption of nitrogenous compounds produced by the *Anabaena*, the relationship is probably far from one-sided. Both Konar and Kapoor (1972) and Peters and Mayne (1974a) suggest that the *Azolla* hairs are in fact secretory. In this context, it is noteworthy that wall ingrowth formation is associated with secretion of solutes into the external environment in angiosperms (e.g. nectaries and many gland cells; Pate and Gunning, 1972). The presence of mucilage within *Azolla* leaf cavities supports this view. However, the absence of mucilage in the leaf cavities of *Anabaena*-free *Azolla* (Duckett *et al.*, 1975b) suggests that the mucilage is probably derived from the *Anabaena*. These cytological observations provide striking morphological support for physiological data (Peters and Mayne, 1974a, b) on the interchange of metabolites between *Azolla* and *Anabaena*. However, it is enigmatic that wall ingrowth development is equally prominent in the absence of the alga (Duckett *et al.*, 1975b). Thus, the situation in *Azolla* is apparently an exception to the general statement that the development of transfer cell morphology coincides with the onset of intensive short distance transport processes (Gunning and Pate, 1969; Pate and Gunning, 1972). It would appear therefore that hair wall ingrowths are a constitutive feature of *Azolla* plants.

Reports of small populations of bacteria sharing the *Azolla* leaf cavity with *A. azollae* (Venkataraman, 1962; Weiringa, 1968) have been confirmed in this investigation. The nitrogen-fixing strains of *Pseudomonas* and *Azotobacter* supposedly isolated from *Azolla* leaf cavities by Bottomly (1920) could possibly have been contaminants since recent studies by

Fisher, Gates and Candler (1980) have shown that only aerobic coryneform bacteria were associated with secretory hairs and *Anabaena* filaments in the leaf cavity of *A. caroliniana*.

*Anabaena azollae* Strasburger is considered to be the only species of alga in symbiotic association with *Azolla* (Moore, 1969; Lumpkin and Plucknett, 1980). It is probable that wide morphological variation within *A. azollae* is also found within the six *Azolla* species, but very little information is available on this subject. The species has sinuous trichomes composed of pale green, bluntly elliptical, bead-like or barrel-shaped cells very often without a sheath (Geitler, 1925; Canabaeus, 1929; Shen, 1960). Each trichome may have three types of cells: vegetative cells, heterocysts and akinetes. Several authors have noted the absence of akinetes (e.g. Lumpkin and Plucknett, 1980), though these are usually found only in the leaf cavities of old *Azolla* leaves (Hill, 1977). Heterocysts and akinetes develop from vegetative cells, apparently in response to external environmental stimuli (Shen, 1960). The difficulty in distinguishing between akinetes and mature heterocysts encountered in this study has also been reported by previous workers (e.g. Hill, 1977).

In this study, vegetative cells of *A. azollae* showed sequential development, becoming larger and changing colour with increasing distance from the rhizome apex. The differentiation of heterocysts and akinetes was also associated with increasing age of *A. azollae* cells. These results support the findings of Hill (1975, 1977) who noted similar changes in the morphology and pattern of cell development in *A. azollae* from *A. filiculoides* plants.

Published reports of cell dimensions in *A. azollae* have been somewhat conflicting. Fjerdingstad (1976) partially reviewed reported values for the dimensions of *A. azollae* and his list is expanded in Table 4.

TABLE 4 - Cell dimensions of *Anabaena azollae* in various *Azolla* species ( $\mu\text{m}$ )

<i>Azolla</i> species	Vegetative cells		Heterocysts		Source
	Width	Length	Width	Length	
<i>A. carolinana</i>	5	8	10		<sup>b</sup> Toni, 1907
<i>A. carolinana</i>	5	8		10	Tilden, 1910
Unknown	4-5,5	5- 9,5	9,5	11,5	Geitler, 1925
Unknown	4-5,4	5- 7,5			Canabaeus, 1929
Unknown	4-5	6- 9,5	6-9,5	9-11,5	Prescott, 1951
<i>A. pinnata</i>	4-5	5- 7	6-7,5	7,5- 8,5	Shen, 1960
<i>A. pinnata</i>	6-8	10-12			Singh, 1977
<i>A. filiculoides</i>	4-6	6-12,5			Hill, 1977
<i>A. filiculoides</i>	5	9	7	11	Lumpkin & Plucknett, 1980
<i>A. filiculoides</i>	3,9-6,1	4,5-13,1	5,8-9,9	9,1-13,2	This study

It is evident from Table 4 that there is considerable variation in the dimensions of both vegetative cells and heterocysts from the three *Azolla* species listed. The cell dimensions quoted by many workers seem to refer only to cells from young and mature leaves, since these dimensions fall into the lower range of the cells measured in this study. It is therefore probable that despite its wide morphological variability, *A. azollae* is the only species of blue-green alga symbiotic with *Azolla*.

Reports that the chronological development of the alga parallels that of the fern (Hill, 1975, 1977; Lumpkin and Plucknett, 1980) are confirmed in this study. This suggests that there might be some form of synchrony in the rate of cell division of the two organisms in the symbiosis. However, the means by which synchrony is achieved are not known at present.

The high heterocyst frequencies in *A. azollae* reported here (up to 33 %), support the observations of other workers (e.g. Peters, 1975; Becking, 1976a; Hill, 1975, 1977; Singh, 1977a) and appear to be a result of the symbiotic association. High heterocyst frequencies, of the same order as those in *A. azollae*, have been reported for symbiotic species of *Nostoc* in the coralloid roots of *Encephalartos* (Caiola, 1975), in the cephalodia of

*Peltigera* species (Hitch and Millbank, 1975) and in the liverworts *Anthoceros* and *Blasia* (Stewart and Rodgers, 1978). On the other hand, free-living species of blue-green algae (e.g. *Anabaena cylindrica*) normally have low heterocyst frequencies (6 to 8 %) (Fogg, 1949; Fay, 1969), unless the alga is grown in nutrient deficient media when heterocyst frequencies may increase up to 12 % (Fogg, Stewart, Fay and Walsby, 1973). The mechanisms whereby the symbiotic alga is stimulated to produce such high heterocyst frequencies are not known at present.

At the ultrastructural level, *A. azollae* possesses a structure typical of many non-gas vacuolate free-living *Anabaena* species and has many features common to the cyanophyta in general (Fogg *et al.*, 1973). In *A. azollae* the outer sheath is poorly developed, with some evidence of a microfibrillar sub-structure, particularly visible at the poles, as reported by Dunn and Wolk (1970). Within the 4-layered cell wall, the most obvious features in *A. azollae* are the numerous membranous thylakoids. These structures contain the photosynthetic pigments and are the sites of photosynthesis (Fogg *et al.*, 1973), suggesting that the alga is capable of autotrophic nutrition. Observations of thylakoid dilation in older cells and heterocysts made here, confirm earlier reports by Grilli (1964) and Lang (1965).

The cytoplasmic inclusions reported for *A. azollae* are identical to those found in other blue-green algae. The close association of polyglucan granules and thylakoid membranes is to be expected since these granules store a glycogen-type photosynthetic product (Pankratz and Bowen, 1963). The scarcity of cyanophycin granules in young cells and their abundance in older cells and akinetes, particularly at cell junctions, confirms the observations of Miller and Lang (1968) and Neumüller and Bergman (1981). These structures contain abundant protein (Fogg, 1951) and have been implicated in the storage of combined nitrogenous products and the proteinaceous pigment cyanophycin (Simon, 1971; Stewart, 1972). Polyhedral bodies were common in young vegetative cells, but comparatively scarce in mature cells, confirming the report by Neumüller and Bergman (1981). These structures contain much or all of the cyanobacterial ribulose-1,5-diphosphate carboxylase (RuDP Carboxylase; the key enzyme in dark CO<sub>2</sub>-fixation via the Calvin Cycle). They are now, on that basis,

considered analogous to the 'carboxysomes' of certain other autotrophs (Stewart and Codd, 1975). The cyanobacterial symbionts of liverworts also contain polyhedral bodies, but do not fix CO<sub>2</sub> or evolve O<sub>2</sub>, and Rodgers and Stewart (1977) have assumed that polyhedral bodies might therefore only have a storage function. The presence of microplasmodesmata at the junctions between heterocysts, vegetative cells and akinetes suggest that there is active transport of solutes between all three cell types.

Akinetes in blue-green algae are produced by differentiation of vegetative cells. As the vegetative cell enlarges, a thickened envelope is formed by localized deposition of dense amorphous and fibrillar material (Lang, 1965). The resulting structure is particularly resistant to adverse conditions, remaining viable for long periods (Fogg *et al.*, 1973).

The six extant species of *Azolla* appear to have identical life cycles, with the exception that sporocarps are formed in tetrads in *A. nilotica* (Demalsy, 1953). The life cycle of *A. filiculoides* described here agrees with earlier reports (Campbell, 1893; Duncan, 1940; Demalsy, 1958; McLean and Ivimey-Cook, 1960). Fertilization of the egg in the archegonium of the megaspore and the embryonic stages of development of the sporophyte take place under water and the young sporophyte floats to the surface only after production of the first and second leaves. Vegetative reproduction is common, a lateral branch simply separates from the main rhizome after the formation of an abscission layer at its base.

Cells or short filaments of *A. azollae* are trapped within developing mega- and microsporocarps. Sporocarp rupture appears to dissipate the algal cells within microsporocarps but algal cells in megasporocarps are retained beneath the indusium cap. Since *Azolla* plants produce sporocarps at the base of axillary branches where a mature leaf forms the involucre, at least a proportion of the algal cells trapped within developing megasporocarps will be akinetes. These akinetes will withstand desiccation (Fogg *et al.*, 1973), while any vegetative cells trapped within the sporocarp would probably die. The symbiotic association is continued when the algal akinetes colonize the new sporophyte apex on germination.

Although no work has been reported on methods of inducing sporocarp formation prior to this study, in *A. filiculoides* it is known to be associated with mat formation (Talley *et al.*, 1977) and summer months in temperate regions (West, 1953; Holst and Yopp, 1979a). Conversely, sporocarp production in *A. pinnata* has only been recorded in the winter months in tropical regions (Shen, 1960; Konar and Kapoor, 1974; Singh, 1977a).

In this study, sporocarp production was primarily controlled by photoperiod, with maximum sporocarp production in the field recorded in summer when daylengths were longer than 12 hours. The low rates of sporocarp production recorded in laboratory studies at photoperiods of between 8 and 12 hours support this finding. This hypothesis is corroborated by the field observations of Holst and Yopp (1979a) that sporocarp production was initiated in June (northern hemisphere) and their comment that photoperiod appeared to be the principal trigger.

Additional evidence for the seasonality of sporocarp production is provided by laboratory experiments investigating the effects of light intensity, temperature and pH. The results indicated that sporocarp formation could not take place during winter because water temperatures, which often drop below 10 °C, were unfavourable. The 'optimum' temperature (25 °C) also indicated that sporocarp production should only occur during the summer months when water temperatures were high. In the laboratory, sporocarp formation was maximal when the water pH was around 9,5 at temperatures of 20 to 28 °C. These conditions are only found in the Hendrik Verwoerd Dam catchment area during the summer months.

During summer the mean midday light intensity in the study area was approximately 115 kLux. Since sporocarp production appeared to be inhibited at light intensities greater than 30 kLux in the laboratory, this indicated that plants of *A. filiculoides* in the lower layers of a mat in the field, shaded from direct sunlight by the overlying layers, were best able to produce sporocarps. This was confirmed by careful examination of thick mats of *A. filiculoides* in the field. Plants in the upper two to three layers were found to lack sporocarps, whilst in the lower layers up to 85 % of the plants had sporocarps. Both in the laboratory and in the

field, *A. filiculoides* only produced sporocarps when the plants were in an established mat, confirming the observation of Talley *et al.* (1977). In the field, sporocarp production only occurred during the summer months October to January, when the interacting effects of photoperiod, water temperature, pH and light intensity were optimal. The reproductive cycle of *A. filiculoides* was found to be closely geared to the seasonal hydrological cycle, accounting for the different sporocarp production times in riverine and farm dam sampling stations.

Prior to this study, no mention of environmental regulation of megaspore germination could be found in the literature. Therefore, the results of these laboratory experiments provide a new insight into the adaptation of *A. filiculoides* to its environment. *Azolla filiculoides* megaspores have a higher resistance to desiccation at low (winter) temperatures than high (mid-summer) temperatures. The temperature recorded at sampling sites in the study area during the spring and autumn months often varied within 2 to 3 degrees of 20 °C. Therefore, the relatively higher percentage germination after desiccation at 20 °C (compared to those at 5 °C and 30 °C) indicated that far more megaspores would be able successfully to withstand desiccation during these months than during winter or mid-summer.

Results of laboratory experiments investigating the effects of photoperiod, light intensity and water temperature support field observations that megaspores germinated predominantly during the summer and early autumn months when daylengths were longer than 10 hours and water temperatures higher than 17 °C. This is borne out by the observation by Holst and Yopp (1979a) that the germination of megaspores in the bottom mud of a pool might be temperature dependent. The very low germination at 10 °C in the laboratory indicated that low (late autumn and winter) temperatures are inhibitory. Similarly, the decreased germination percentage that was recorded when water temperatures were increased from 20 to 30 °C indicated that megaspores are sensitive to high water temperatures, though this was less than their sensitivity to desiccation at high temperatures.

Megaspore germination in the laboratory occurred over a wide range of water temperatures though high water temperatures were clearly as inhibitory as desiccation at these temperatures (e.g. 35 °C). The very low percentage

germination of 1 % at 10 °C in these experiments (compared with previous results: 6 %), may have been due to experimental error or to the use of immature megaspores. An interesting feature of this series of experiments was the slight increase in 'optimum' temperature from 22,5 to 25 °C with light intensities greater than 15 kLux. The high optimum temperatures recorded (22,5-25 °C) indicated that maximum germination should take place in summer when water temperatures were high. However, significant germination percentages were recorded at temperatures (12,5-15 °C) that would normally be recorded in late spring or early autumn.

Due to the geological and pedological characteristics of the study area, most of the streams and rivers are often highly turbid. Thus, the ability of megaspores to germinate at low light intensities (0,5 kLux) is an advantage in this habitat. High light intensities (60 kLux) were clearly inhibitory, reducing the percentage germination to below that recorded at 0,5 kLux. The fact that maximum germination occurred at 30 kLux indicated that a higher percentage germination would be recorded under lightly shaded (or slightly turbid) conditions in the field.

The results of experiments investigating the interacting effects of water temperature and pH clearly demonstrated that both low and high water temperatures inhibited megaspore germination. The 'optimum' temperature of 20 °C indicated that maximum germination should occur during the summer months, confirming the results obtained in the previous experiments. An interesting feature was the very low germination recorded at pH 9,0. In the study area, the pH of the surface waters at the different sampling sites varied between 8,2 and 9,4 while that of the bottom waters varied between 6,1 and 8,5. Thus, the surface waters were at a pH unfavourable for maximum megaspore germination. However, the bottom waters had a pH range which spanned the range at which maximum germination was recorded in the laboratory. Thus, the normal procedure whereby mature *Azolla* megaspores sink to the bottom of the pool or stream before germinating, allows the megaspores to take advantage of a more favourable environment for germination.

Turbulence was also shown to markedly affect megaspore germination. This may have been due to some form of physical damage to the developing

sporophyte under the indusium cap, probably abrasion. Microscopic examination of non-germinated megaspores at the end of each treatment revealed that many had lost the indusium cap and/or had lost one or more 'floats', particularly in those treatments receiving moderate to severe turbulence, supporting the hypothesis that physical damage had prevented germination. However, some of those megaspores which became damaged during the experimental treatment may not have been sufficiently mature (i.e. with a hardened perispore wall) to withstand the turbulence. Despite this possibility, it is evident from the results that the megaspores possess a degree of resistance to disturbance, permitting germination (albeit delayed) even under conditions of severe turbulence. Successful germination, under conditions of moderate to severe turbulence, no doubt accounts for the spread of *A. filiculoides* from the study area to a point some 450 km down the Orange River in the Upington area (see Figure 8).

The size of *A. filiculoides* populations at the twelve sampling sites was primarily dependent on the hydrological regime at each site. The presence of a standing body of water was essential for mat development and the long pools present in most streams near the Hendrik Verwoerd Dam provided ideal habitat for the fern. Those plants in stream or riverine sites were particularly susceptible to flood-induced wash-out, while plants in farm dams were more protected. During period of low flow, *A. filiculoides* populations increased markedly at all sites, both by vegetative propagation and through the germination of sporocarps. *Azolla* megaspores, able to over-winter and survive desiccation in the mud of dried-out streams, re-colonized the water once the pool had been refilled. During periods of mild and severe flooding the fragile *Azolla* plants were easily fragmented and dispersed. Sporocarps on the other hand did not fragment easily and thus served both as a source of infestation for localities further downstream and a means of re-colonizing flood-cleared pools. The flooding therefore gave rise to a seasonal fluctuation in the population density of *A. filiculoides*, and at each site the system may therefore be considered to be self-regulatory.

## CHAPTER 5

### ENVIRONMENTAL REGULATION OF GROWTH AND NITROGENASE ACTIVITY IN THE SYMBIOTIC ASSOCIATION

#### 5.1 INTRODUCTION

Because *Azolla filiculoides* floats freely on the water surface, it can be influenced by physical and chemical factors from both the air and water phases. In general, however, the fern behaves in much the same way as most green plants, in that it has optimal temperature, light and pH requirements (Ashton and Walmsley, 1976). *Azolla filiculoides* plants are ideal test organisms for laboratory studies since they are small, have an aquatic habit and show rapid vegetative reproduction enabling the use of clones. The endosymbiotic blue-green alga is able to fix atmospheric nitrogen and can supply the nitrogen requirements of both organisms in the symbiosis (Peters, Toia, Evans, Crist, Mayne and Poole, 1980). Measurements of nitrogenase activity (nitrogen fixation rates) can therefore provide useful supplementary information to growth analyses.

Prior to the initiation of this study, very little quantitative work concerning the effects of environmental factors on the growth of *A. filiculoides* had been reported in the available literature, though several reports have appeared on other species of *Azolla*, particularly *A. pinnata* and *A. caroliniana*. A notable feature of the rather scattered literature is the variety of units used to express irradiance or light intensity. To facilitate the conversion of the different units to those used in this study (kLux), a conversion table is included (Table 5).

The only quantitative studies carried out on *A. filiculoides* were those of Ahmad (1941, 1943), Kellar and Goldman (1979) and Peters *et al.* (1980). Ahmad (1941) reported maximum growth rates of *A. filiculoides* occurred in the range 0,5 to 2 kLux. Peters *et al.* (1980) disagreed, reporting that growth rates increased with increasing photoperiod up to a maximum at 16 hours daylength, and that growth rates also increased with increasing light intensity to a maximum at a photon flux density of  $400 \mu\text{moles.m}^{-2} \cdot \text{sec}^{-1}$ , with no inhibition of growth at higher light intensities. These authors also recorded that nitrogenase activity was maximal at lower light intensities than maximum growth rates.

TABLE 5 - Irradiance conversion factors for photosynthetically available radiation (P.A.R.). These data are adapted from northern hemisphere data provided by Harris (1978) and Peters *et al.* (1980)

---

Full sun	=	360 W.m <sup>-2</sup> P.A.R.
	=	9 000 ft. c.
	=	96 kLux
	=	0,53 cal.cm <sup>-2</sup> .min <sup>-1</sup> P.A.R.
	=	1 680 μE.m <sup>-2</sup> .sec <sup>-1</sup> P.A.R.
	=	1,3 x 10 <sup>13</sup> ergs.m <sup>-2</sup> .hr <sup>-1</sup> P.A.R.
	=	1 680 μmoles of photons.m <sup>-2</sup> .sec <sup>-1</sup> P.A.R.
P.A.R. 1 W.m <sup>-2</sup>	=	4,66 μE.m <sup>-2</sup> .sec <sup>-1</sup> = 0,0168 E.m <sup>-2</sup> .hr <sup>-1</sup>
1 ft. c.	=	0,1867 μE.m <sup>-2</sup> .sec <sup>-1</sup>
1 kLux	=	17,5 μE.m <sup>-2</sup> .sec <sup>-1</sup>
1 cal.cm <sup>-2</sup> .min <sup>-1</sup>	=	3 169 μE.m <sup>-2</sup> .sec <sup>-1</sup>

---

Ahmad (1941, 1943) found that at temperatures above 20 °C in the laboratory, *A. filiculoides* plants did not grow until the light intensity exceeded 0,5 kLux, with the 'optimum' temperature being 22 °C for the low light intensities used. However, Peters *et al.* (1980) showed that *A. filiculoides* had a lower temperature optimum (25 °C) than two tropical species (*A. mexicana* and *A. pinnata*), which had temperature optima of 30 °C. A temperature of 40 °C caused a marked reduction in rates of growth and nitrogenase activity. These authors also reported that *A. filiculoides* plants showed similar high rates of growth and nitrogenase activity when cultured in the pH range 5 to 8. A pH of 9 caused a marked decrease in rates of growth and nitrogenase activity.

Kellar and Goldman (1979) reported decreased rates of growth and nitrogen fixation in crowded natural populations of *A. filiculoides* var. *rubra* in New Zealand. Periods of wind and wave action not only dispersed and fragmented the *A. filiculoides* plants, but also damaged frond apices and resulted in lower rates of nitrogen fixation (Kellar and Goldman, 1979).

During the course of the research leading up to the preparation of this thesis, some results were published (Ashton, 1974, 1977; Ashton and Walmsley, 1976). These publications will not be reviewed here as their content is included in the research findings of this thesis. Most ecological and physiological studies of the genus *Azolla* have involved aspects of its relationship with the associated alga *Anabaena azollae*. These features will be treated in Chapters 7 and 8 of this dissertation. This chapter deals solely with the effects of environmental factors on the growth and nitrogenase activity of the symbiotic association. These findings are used to account for the limited distribution and the patterns of growth and nitrogen fixation shown by *A. filiculoides* in the temperate environment of the Hendrik Verwoerd Dam catchment area.

The specific aims of this investigation were:

- (a) to investigate the regulatory effects of various environmental factors and to characterize the optimal environmental conditions for growth and nitrogen fixation by the *A. filiculoides*-*A. azollae* association, and
- (b) to determine the advantages (if any) of mat formation with regard to the symbiotic association's ability to survive unfavourable conditions.

## 5.2 MATERIALS AND METHODS

### 5.2.1 Measurement of nitrogen fixation rates

Schollhorn and Burris (1966) and Dilworth (1966) independently observed the inhibitory effects of acetylene on nitrogen fixation by extracts of *Clostridium pasteurianum*. Dilworth (1966) also found that the acetylene was reduced to ethylene in a reaction analogous to the reduction of nitrogen gas to ammonia. The application of this reaction to a sensitive gas chromatographic assay procedure for nitrogen-fixing activity was proposed by Hardy and Knight (1967). Subsequently, several workers (e.g. Koch, Evans and Russel, 1967; Stewart, Fitzgerald and Burris, 1967, 1968; Hardy, Holsten, Jackson and Burns, 1968) successfully used acetylene reduction as an assay for nitrogenase activity. Since the

original proposal of Hardy and Knight (1967), the acetylene reduction assay has undergone extensive development. This has resulted in an extremely sophisticated technique with numerous applications in biology. The assay is "... sensitive, universal, specific, rapid, simple, economical and quantitative" (Hardy *et al.*, 1968).

The acetylene reduction assay does not measure the actual quantity of nitrogen fixed. Rather, the method provides an accurate, though indirect, index of the activity of a nitrogen-fixing system, in terms of the ability of that system to fix atmospheric nitrogen under a given set of environmental conditions. It is often necessary, however, to relate the results obtained with the acetylene reduction assay to actual quantities of nitrogen fixed. This can be accomplished by parallel determinations of the amount of nitrogen fixed and acetylene reduced under the same conditions. The molar ratio of ethylene produced to ammonia produced can then be used to convert acetylene reduction assay results to actual values of nitrogen fixed. The reduction of 1 mole of nitrogen to 2 moles of ammonia requires the transfer of 6 electrons and the reduction of 1 mole of acetylene to 1 mole of ethylene requires only 2 electrons (Stewart *et al.*, 1967). Theoretically, therefore, the reduction of acetylene to ethylene should occur 3 times as fast as the reduction of nitrogen to ammonia, with a molar ratio of ethylene to ammonia equalling 1,5 (Stewart *et al.*, 1967). However, published data indicate that this ratio varies considerably between different organisms. This may be due, in part, to the relatively insensitive methods such as Kjeldahl analysis and mass spectrometric analysis of <sup>15</sup>N-incorporation which are used to obtain direct values of any nitrogen fixed (Hardy *et al.*, 1968).

#### 5.2.1.1 Acetylene reduction assays

For the routine analysis of nitrogenase activity in the laboratory, 3 to 5 fronds of acclimatized *Azolla filiculoides* were gently blotted dry, weighed and then placed in 25 ml capacity reaction flasks fitted with rubber septa containing 5 ml of the appropriate nutrient solution. Each flask was then flushed for 4 minutes with a mixture of 0,1 atmospheres acetylene (purified by bubbling through concentrated H<sub>2</sub>SO<sub>4</sub> followed by drying over CaCl<sub>2</sub>) and 0,9 atmospheres of a mixture of argon, oxygen and carbon dioxide in the ratio 77,665 : 22,3 : 0,035. The gas mixture ratio was controlled with a

Matheson model 665 gas proportioner fitted with a needle valve control. The reaction flasks were then incubated in the required environmental conditions for an appropriate period, usually 20 to 40 minutes.

In the field, weighed samples of blotted *Azolla* material (approximately 2,0 g per analysis) were placed in the barrels of 50 ml glass syringes. In each case the syringe plunger was then inserted and as much air as possible expelled without squashing the plants. A hypodermic needle was then fitted to the syringe, the needle inserted through the rubber septum of a flask containing acid-scrubbed acetylene and 5 ml of the purified acetylene drawn into the barrel of the syringe. The needle was then withdrawn from the acetylene flask, and a further 45 ml of air drawn into the syringe barrel. The needle was then sealed by spearing a rubber stopper. The syringes containing samples were then incubated *in situ* for 20 to 40 minutes. After incubation, each syringe was thoroughly shaken to mix the gases within the barrel, the stopper was removed and the needle quickly inserted through the septum of an evacuated 25 ml capacity flask. A volume (25 ml) of the gas phase was injected into the flask which was then transported to the laboratory. Each treatment was replicated three times in both field and laboratory experiments. Samples of the acid-scrubbed acetylene were also analysed for ethylene contamination.

In the laboratory, triplicate 50  $\mu$ l samples of the gas phase in each flask were withdrawn and analysed for their ethylene content by gas chromatography. The instrument used was a Perkin-Elmer 990 gas chromatograph equipped with a flame ionization detector and fitted with a 2 metre long, 2 mm internal diameter stainless steel column containing 80/100 mesh Porapak N. All analyses were carried out at a column temperature of 60 °C with inlet and detector temperatures of 120 °C. High purity nitrogen (African Oxygen Co.) served as the carrier gas at a flow rate of 35 ml per minute. In time course studies, the gas samples removed for analysis at each interval were replaced with an equivalent volume of the appropriate nutrient solution.

The gas chromatograph was calibrated with serial dilutions of a standard mixture of acetylene (0,1 atmospheres) and ethylene ( $2 \times 10^{-3}$  atmospheres) in argon. A typical chromatogram is shown in Figure 36. The retention

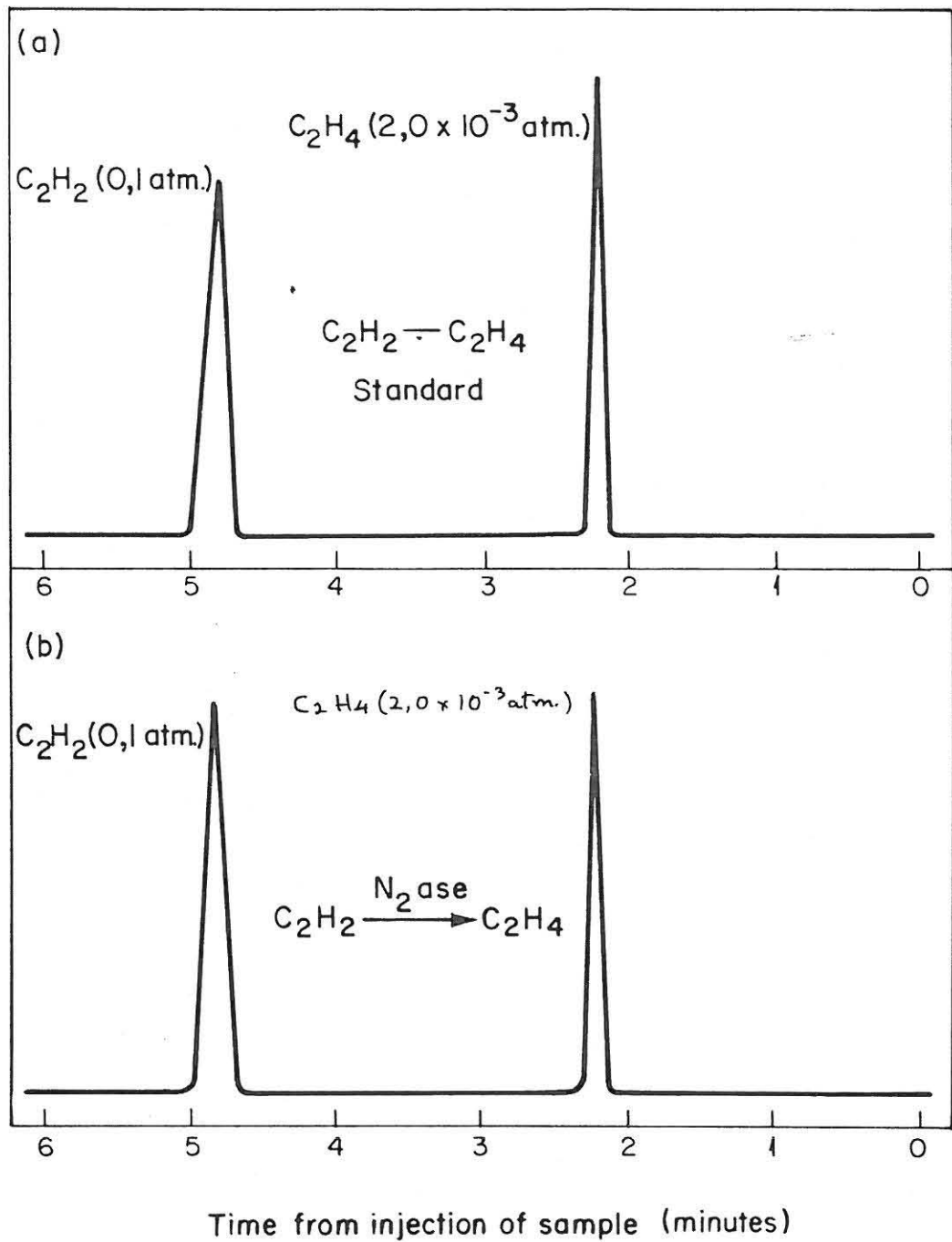


FIG. 36 *Typical chromatograms of : (A) 200  $\mu$ l of a known mixture of  $C_2H_2$  (0,1 atm.),  $C_2H_4$  ( $2,0 \times 10^{-3}$  atm.) and Ar to 1 atm., and (B) 200  $\mu$ l of the gas phase from an incubation after nitrogenase - catalysed reduction of 0,1 atm. of  $C_2H_2$ .*

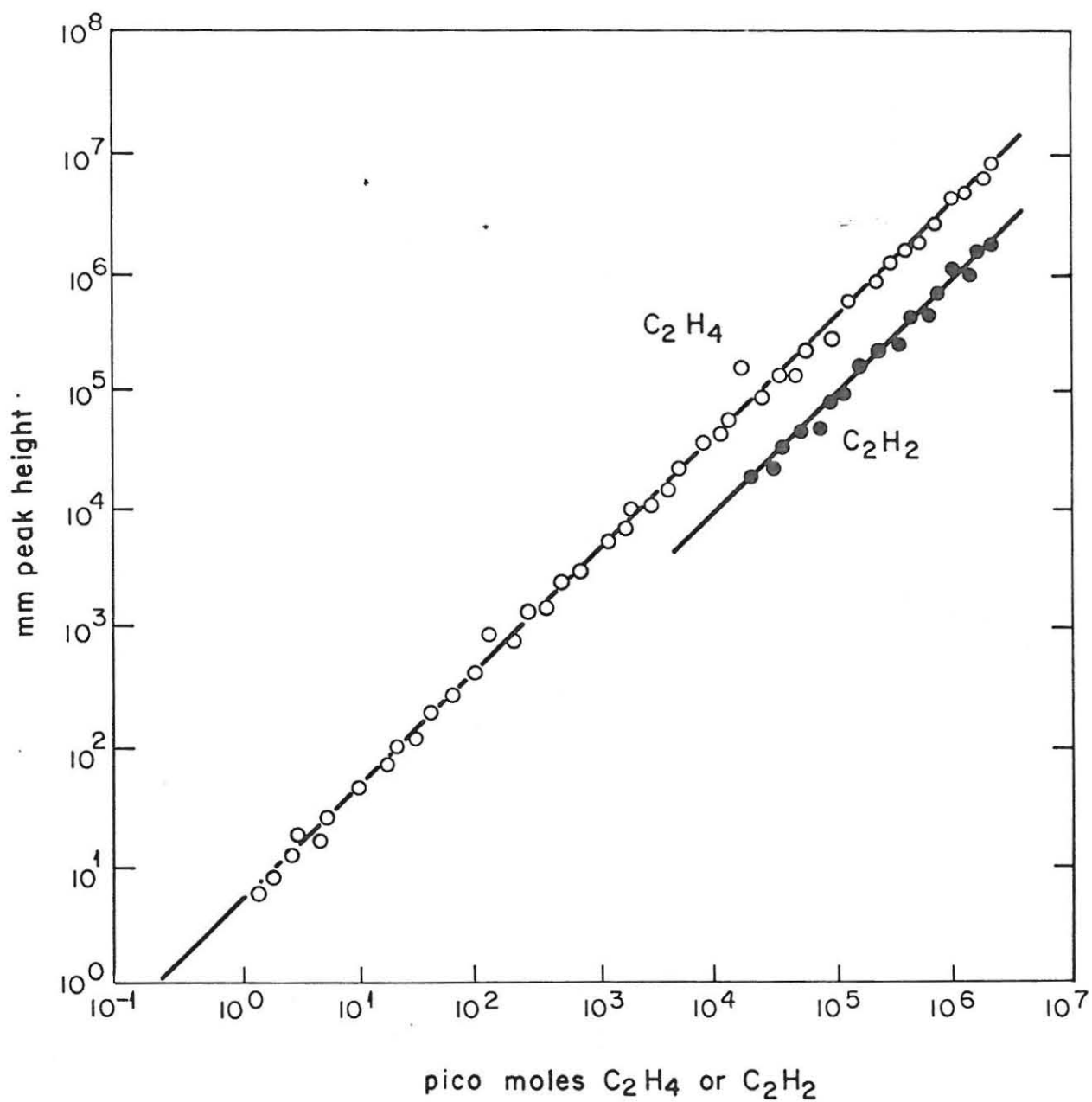
times of the ethylene and acetylene peaks were 2,2 minutes and 4,8 minutes respectively and the excellent separation and resolution of the individual peaks is clearly shown. A standard calibration plot of peak height against the ethylene or acetylene content of injected samples (Figure 37) demonstrates the linear response and sensitivity of the assay since less than  $10^{-11}$  moles of ethylene could be detected per injected sample of 50  $\mu\text{l}$ . As noted by Hardy *et al.* (1968), the ethylene content of a sample can be accurately calculated from the calibration plot. Alternatively, the 'built-in' internal standard acetylene can be used, though this is less accurate. Before and after each analytical run of a batch of samples, a range of standard acetylene + ethylene standards were analysed to check the accuracy of the calibration.

Abiological ethylene production caused by the reaction of metabolic poisons used to stop the nitrogenase activity, reacting with rubber septa, has caused serious errors in earlier work (Schell and Alexander, 1970; Thake and Rawle, 1972). For this reason a sample of the gas phase was always withdrawn immediately after incubation and stored before analysis. This precluded the possibility of abiological ethylene production.

#### 5.2.1.2 Kjeldahl analysis

A mass spectrometer was not available during this study, consequently Kjeldahl analysis was used to obtain a direct estimate of the rate of nitrogen fixation under different environmental conditions. Essentially, the method was identical to that employed for routine determinations of plant nitrogen content (Section 2.4.1 c) except that larger quantities of plant material and reagents were used and analysis was carried out in 800 ml capacity Kjeldahl flasks.

The plant material was acclimatized on nitrogen-free media under the appropriate conditions for one week prior to each experiment. Half of the acclimatized plants were harvested at the beginning of the experimental period and the remainder were harvested 24 hours later. Both sets of harvested plants were weighed, dried at 105 °C for 48 hours, reweighed and analysed for nitrogen content. The difference in nitrogen content per unit weight was taken as the quantity of nitrogen fixed.



**FIG. 37** *Standard calibration plot of peak height versus acetylene (C<sub>2</sub>H<sub>2</sub>) and ethylene (C<sub>2</sub>H<sub>4</sub>) concentrations to show linearity and sensitivity of response.*

### 5.2.2 Routine measurements of growth and nitrogenase activity in the field

For purposes of estimating the growth of *A. filiculoides* at stations 9 and 10, the frame apparatus described in Section 2.5.1 was used. Each month, weighed samples (approximately 1,0 g) of washed and blotted *Azolla* plants were placed in each of 10 dishes and the apparatus fastened to a stake in full sunlight. One week later, the plants were removed, blotted dry, reweighed and stored in numbered plastic bags on ice. On return to the laboratory, the plant samples were thawed and analysed for nutrient content. These procedures were followed over the period February 1971 to May 1974. Growth rates were calculated each month, using the procedure described in Section 2.5.2.2 and results were expressed as mean relative growth rates ( $\overline{\text{RGR}}$ ).

Monthly measurements of acetylene reduction rates were made at stations 9 and 10 from February 1972 to February 1974. At each site, samples of *A. filiculoides* were incubated as described in Section 5.2.1.1 from approximately 11h00 to 12h00 local time. Occasional diel estimates of nitrogenase activity were carried out so as to span a 24 hour cycle, starting and ending at approximately 04h00.

### 5.2.3 Laboratory studies

In the laboratory, *A. filiculoides* plants were grown in large walk-in constant environment rooms (Section 2.5.2.1) on the (N-free) AZOLLA-I medium described in Section 6.4.3. Wherever possible, all *A. filiculoides* material was acclimatized for three weeks at the required conditions (Section 2.5.2.2) before rates of growth and nitrogenase activity were measured. In some experiments, however, the environmental conditions used were so unfavourable that the plants regressed rapidly. In these cases, rates of growth and nitrogenase activity were measured after four to six days exposure to the conditions under investigation.

In this study, the effects of temperature, photoperiod, light intensity, pH and salinity on the rates of growth and nitrogenase activity of *A. filiculoides* were investigated. In each experiment, one environmental variable was tested at several different levels while the other variables were held constant. In initial experiments, diurnal and nocturnal tempe-

ratures were held constant. However, in order to simulate the diel temperature fluctuations recorded in the field, a ten degree temperature differential between diurnal and nocturnal temperatures was introduced in later experiments. This was accomplished automatically, by adjusting the appropriate selectors on the constant environment rooms. In all cases the thermoperiod was adjusted to coincide with the photoperiod. For convenience of notation, photoperiods were indicated as day/night e.g. 16L/8D.

Diurnal and nocturnal temperatures were written with the diurnal temperature first, e.g. 25/15 °C. Rates of growth and nitrogenase activity were measured as described in Sections 2.5.2.2 and 5.2.1.1, respectively. For the investigation of salinity effects, the AZOLLA-I medium was supplemented with analytical grade NaCl to give the required salinity level. Osmotic effects were obtained by using varying amounts of polyethylene glycol (PEG 6000) in addition to the basal medium. The appearance of toxicity symptoms (chlorosis and necrosis) in *A. filiculoides* was used to differentiate salinity effects from purely osmotic effects. The quantities of PEG 6000 required to achieve the different osmotic pressures were calculated from the formula given in Documenta Geigy (1962):

$$P = \left(\frac{R \cdot T}{M}\right) \cdot C \quad (12)$$

where:

P = osmotic pressure in atmospheres

R = gas constant (0,08205 litre atmospheres)

T = absolute temperature (K) = (°C + 273.16)

M = molecular weight of solute (in this case, 6000 for PEG 6000)

C = concentration of solute (g.l<sup>-1</sup>)

Rates of growth and nitrogenase activity were measured as described in Sections 2.5.2.2 and 5.2.1.1, respectively.

### 5.3 RESULTS

#### 5.3.1 Growth and nitrogenase activity in the natural environment

In both the farm dam (station 9) and riverine (station 10) sites, *Azolla filiculoides* was present throughout the study and showed similar patterns of variation in  $\overline{\text{RGR}}$  (Figure 38). Values of  $\overline{\text{RGR}}$  were low to very low during the mid-winter months June-July and the plants were frost-hardy, withstanding short, occasional periods of below-freezing temperatures (absolute minimum of  $-8^{\circ}\text{C}$ ) without apparent harm. In early summer, growth increased logarithmically reaching a maximum in December.  $\overline{\text{RGR}}$  values decreased in late summer and autumn, reaching a minimum ( $0,2\ \%\cdot\text{day}^{-1}$ ) during July. Each year, the annual cyclic patterns followed by both air and water temperatures were very similar. Variations in water pH at stations 9 and 10 differed from each other and varied from year to year (Figure 38).

All *A. filiculoides* plants grown in full sunlight remained red in colour throughout the study, while plants grown in shaded marginal areas were yellowish-green to dark green.

At both sampling sites, the  $\overline{\text{RGR}}$  of *A. filiculoides* was strongly correlated with temperature, in particular average air temperature and average minimum air temperatures (Table 6). The correlation between  $\overline{\text{RGR}}$  and water pH was not significant at acceptable levels ( $P = 0,05$ ).

TABLE 6 - Correlations between the mean relative growth rate ( $\overline{\text{RGR}}$ ) of *Azolla filiculoides* at stations 9 and 10 and some environmental parameters (n = 40 for all data sets)

Parameter	$r^2$ value	
	Station 9	Station 10
average air temperature	0,8863*	0,8929*
average minimum air temperature	0,8706*	0,8853*
average maximum air temperature	0,8113*	0,8125*
surface water temperature	0,7296*	0,8060*
surface water pH	0,0457**	0,1846**
hours of sunlight	0,7989*	0,8184*

\* = highly significant ( $P \ll 0,001$ )

\*\* = not significant ( $P \gg 0,05$ )

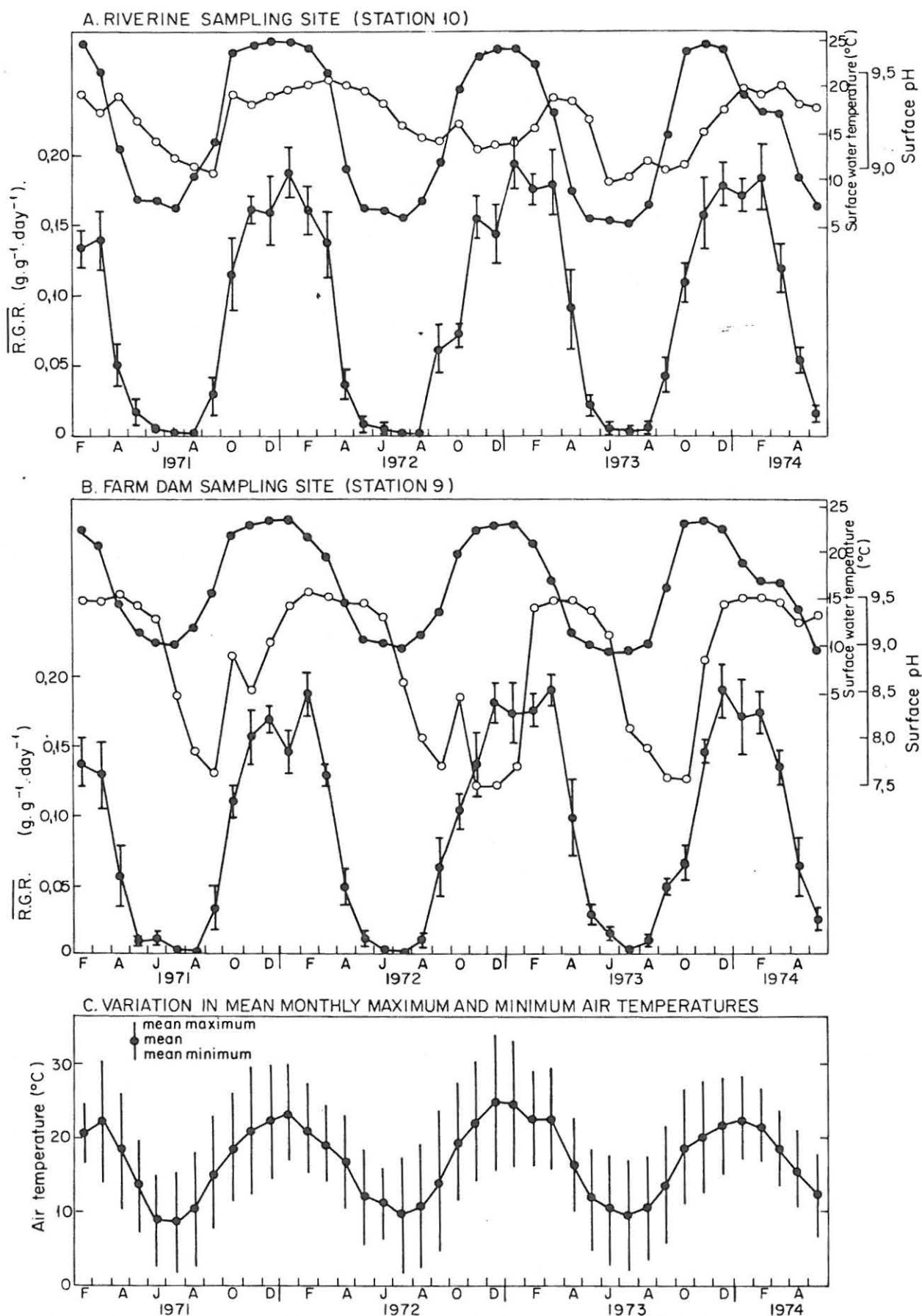


FIG. 38 Comparison of the influence of surface water temperature (●—●) and pH (○—○) on the mean relative growth rate ( $\overline{\text{R.G.R.}}$ ) of *Azolla filiculoides* growing at (A) a riverine station with continuous inflow, and (B) a farm dam with intermittent inflow, from February 1971 to May 1974. (Figure C shows the variation in air temperature during this period). [Vertical bars indicate two standard errors].

At both stations, the  $\overline{\text{RGR}}$  of *A. filiculoides* (Y) developing between July and January of each year could be estimated as logarithmic functions of the mean air temperature (X):

$$Y_{\text{st.9}} = -0,4398 + 0,1905 \ln X \quad (r^2 = 0,893; \quad n = 21) \quad (13)$$

$$Y_{\text{st.10}} = -0,4857 + 0,2076 \ln X \quad (r^2 = 0,875; \quad n = 21) \quad (14)$$

The variations in standing crop (biomass per square metre) and nitrogenase activity at the two sampling sites were quite different (Figure 39). At the riverine site (station 10), biomass followed an annual cyclic pattern, with minima in February-March, increasing to a maximum in December. At station 9, however, biomass remained high for most of the year with lowest values recorded in February and March each year.

Nitrogenase activity at station 10 also followed an annual cyclic pattern with maxima in October-November and lowest values were recorded between February and mid-winter (June-July). At station 9, nitrogenase activity was erratic, and low values were recorded both in mid-summer and mid-winter.

At both sampling sites, the biomass and nitrogenase activity of *A. filiculoides* was poorly correlated with most environmental parameters (Table 7). The only significant correlation found was between nitrogenase activity at station 10 and water temperature. All other correlations were not significant.

In the field, the nitrogenase activity of *A. filiculoides* increased after sunrise (05h00), reaching a peak at midday (12h00), but declined during the afternoon (14h00) (Figure 40). Nitrogenase activity decreased during the night, reaching a minimum shortly before sunrise. Rates of ethylene production between 10h00 and 14h30 did not differ significantly from each other ( $P \gg 0,05$ ), and accounted for approximately 50 % of the total diel ethylene production. From these observations it was evident that on calm clear days nitrogenase activity was significantly higher ( $P \ll 0,001$ ) than that recorded on cloudy overcast days (Figure 40).

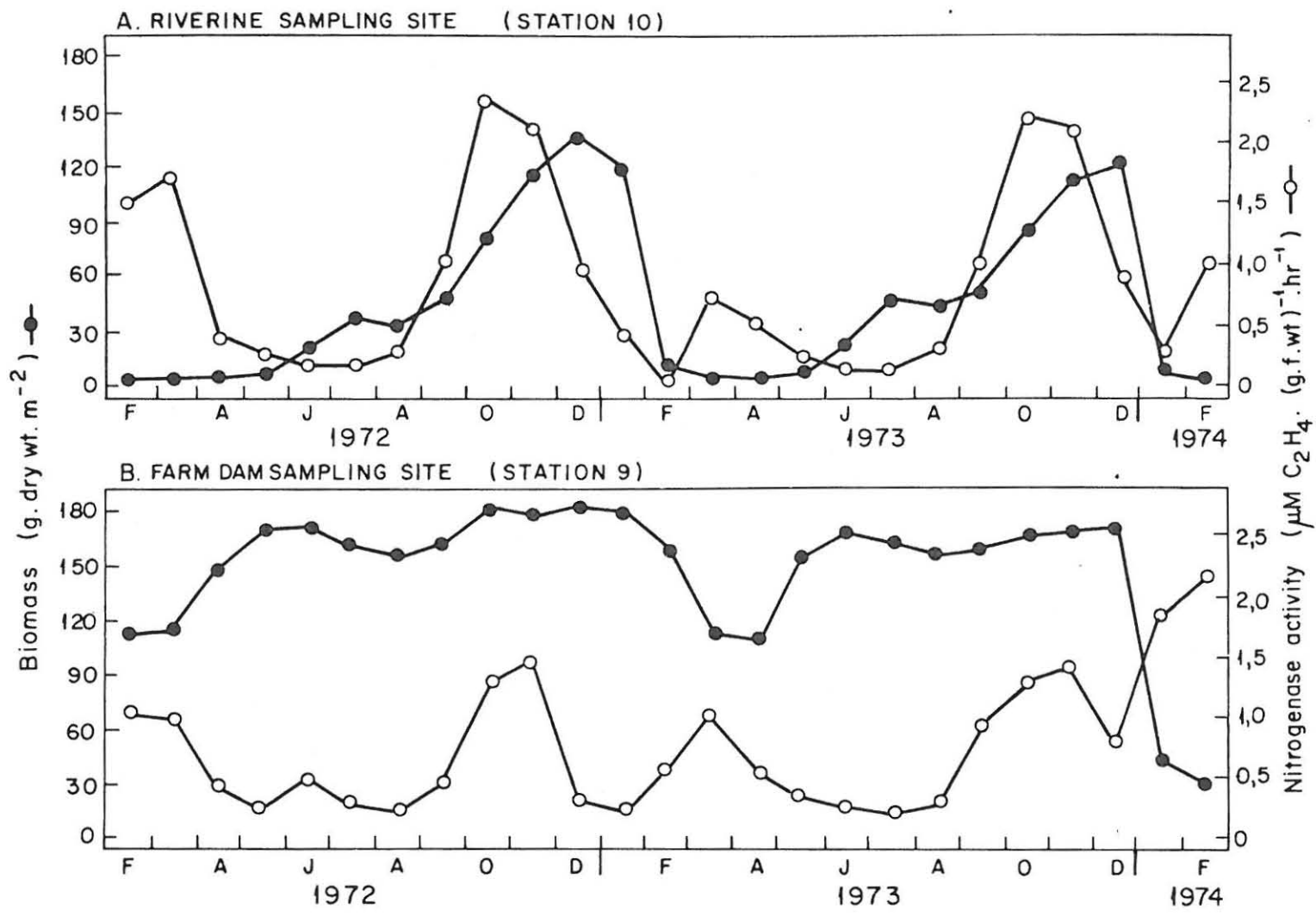


FIG. 39 Seasonal variation in biomass and nitrogenase activity of *Azolla filiculoides* at (A) a riverine sampling site with continuous inflow, and (B) a farm dam with intermittent inflow, from February 1972 to February 1974.

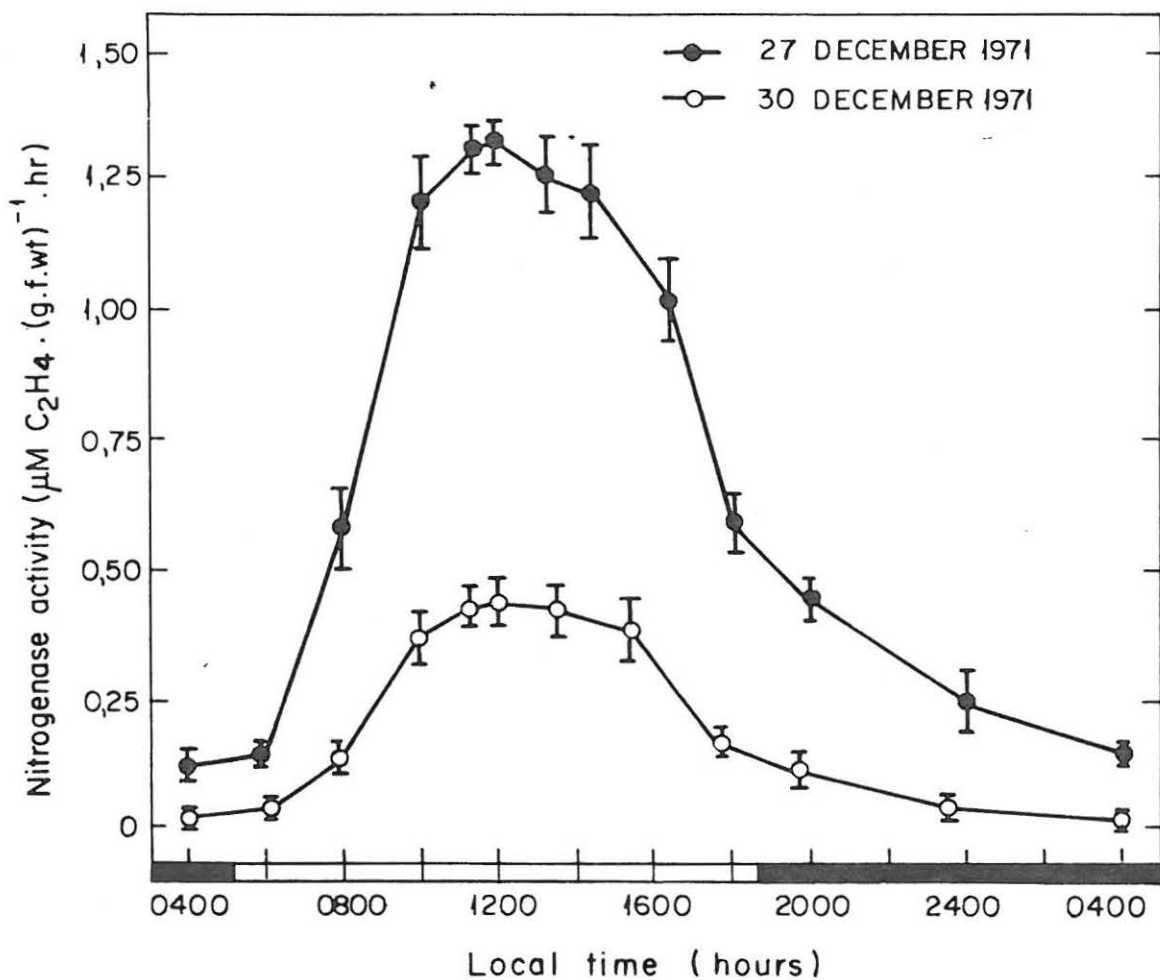


FIG. 40 *Diel variation in nitrogenase activity of Azolla filiculoides in the field. (27 December : clear, calm day; 30 December : cloudy and overcast for second successive day). [Vertical bars indicate two standard errors ; shaded bar shows light and dark periods].*

TABLE 7 - Correlations between biomass and nitrogenase activity (EPR) of *Azolla filiculoides* at stations 9 and 10 and some environmental parameters. (n = 25 for all data sets)

Parameter	r <sup>2</sup> values			
	Station 9		Station 10	
	Biomass	EPR	Biomass	EPR
average air temperature	0,0701	0,2959	0,1553	0,2068
average minimum air temperature	0,1540	0,3639	0,1598	0,1683
average maximum air temperature	0,0148	0,2074	0,2720	0,2329
surface water temperature	0,0003	0,2961	0,2881	0,4499*
surface water pH	0,2719	0,0275	0,2165	0,0396
hours of sunlight	0,0308	0,3334	0,3397	0,2786

\* = significant (0,02 > P > 0,01)

Preliminary experiments based on field observations indicated that light intensity was important in regulating both  $\overline{\text{RGR}}$  and nitrogenase activity in *A. filiculoides*. Field experiments showed that  $\overline{\text{RGR}}$  and nitrogenase activity displayed maxima in 50 % sunlight (Figure 41). Above and below this level, both  $\overline{\text{RGR}}$  and nitrogenase activity declined. At 75 % sunlight,  $\overline{\text{RGR}}$  was slightly (though not significantly) greater ( $P > 0,05$ ) than at 25 % sunlight. At 12½ % sunlight,  $\overline{\text{RGR}}$  was significantly ( $P < 0,01$ ) lower than at higher light intensities. At 50 % sunlight, the nitrogenase activity was not significantly ( $P > 0,05$ ) higher than 25 % or 75 % sunlight. Nitrogenase activities in full sunlight (100 %) and at 12½ % sunlight were not significantly ( $P > 0,05$ ) different from each other. During these experiments, the midday full sunlight light intensity varied between 93 and 109 kLux (average 105 kLux).

At light intensities up to and including 50 % of full sunlight it was observed that the *A. filiculoides* plants turned bright green, whereas at greater than 75 % of full sunlight, *A. filiculoides* remained red in colour.

### 5.3.2 Laboratory studies

#### (a) Light intensity

The results obtained from field investigations on the effects of light intensity were verified in a series of laboratory experiments (Figure 42).

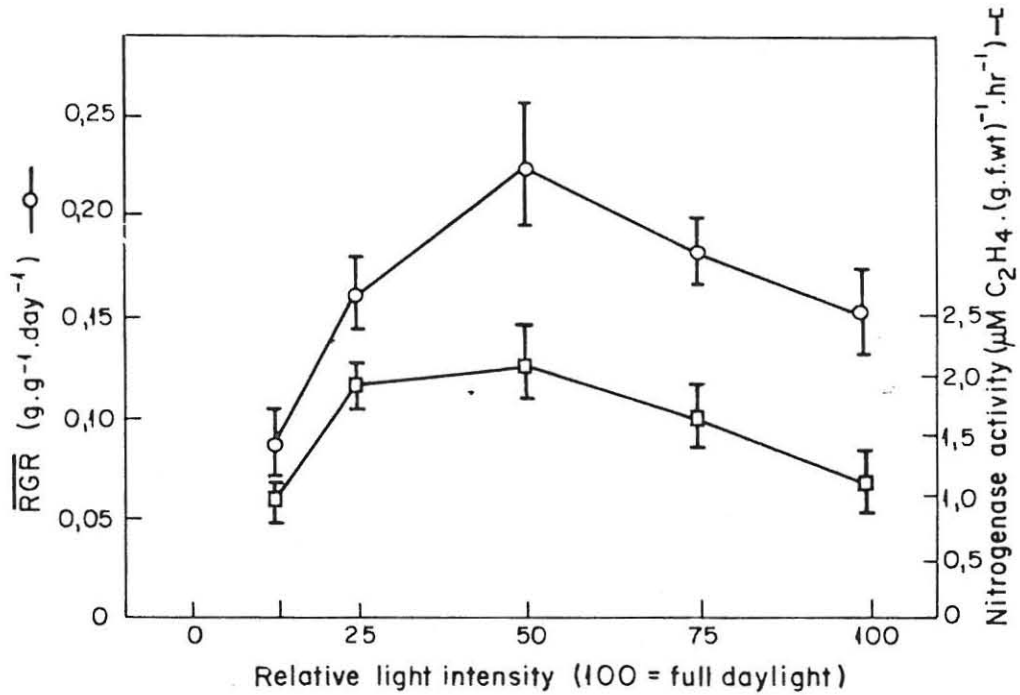


FIG. 41 *The effect of different light intensities on the mean relative growth rate ( $\overline{RGR}$ ) and nitrogenase activity of Azolla filiculoides in the field. (Full daylight averaged 95 kLux at midday). [Vertical bars indicate two standard errors].*

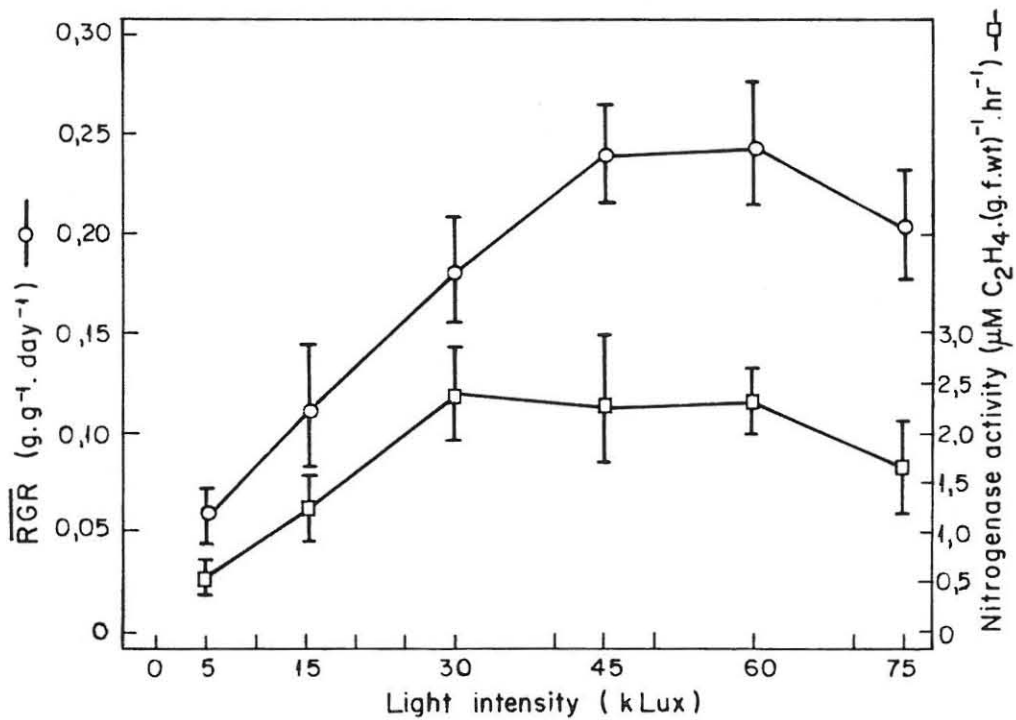


FIG. 42 *The effect of different light intensities on the mean relative growth rate ( $\overline{RGR}$ ) and nitrogenase activity of Azolla filiculoides in the laboratory. (16 hour day; 25°C; pH 8,0). [Vertical bars indicate two standard errors].*

These showed that  $\overline{\text{RGR}}$  increased with increasing light intensity from 5 to 45 kLux, remained constant up to 60 kLux and then declined. The observed values of  $\overline{\text{RGR}}$  at 45 and 60 kLux were significantly greater ( $P < 0,01$ ) than those at lower light intensities and slightly (but not significantly) greater ( $P > 0,05$ ) than those at 75 kLux. Nitrogenase activity increased with increasing light intensity from 5 to 30 kLux, remained more or less constant to 60 kLux and declined at 75 kLux. Nitrogenase activity at 30 to 60 kLux was significantly greater ( $P < 0,01$ ) than those at lower light intensities and only slightly (but again not significantly) greater ( $P > 0,05$ ) than the values recorded at 75 kLux.

On a time-course basis, the results from the field experiment with different light intensities showed that *A. filiculoides* plants required at least 16 days before they became acclimatized to a particular treatment (Figure 43).

(b) Constant temperature

The effects of constant temperature on the  $\overline{\text{RGR}}$  of *A. filiculoides* were studied at three different light intensities (15, 30 and 45 kLux) and over the temperature range 5 °C to 45 °C (Figure 44). With increasing light intensity, the values of  $\overline{\text{RGR}}$  increased at all temperatures up to 35 °C. At 40 °C the differences in  $\overline{\text{RGR}}$  values at the three light intensities were not distinguishable. The  $\overline{\text{RGR}}$  was zero in plants kept at 45 °C. The 'optimum' constant temperature giving rise to maximum  $\overline{\text{RGR}}$  values increased from 22,5 °C at 15 kLux to 27,5 °C at 45 kLux. However, this difference was not statistically significant ( $0,10 > P > 0,05$ ). The difference in  $\overline{\text{RGR}}$  values at temperatures between 20 °C and 30 °C at 45 kLux was not significant ( $0,10 > P > 0,05$ ). However, at light intensities of 15 kLux and 30 kLux,  $\overline{\text{RGR}}$  values at 30 °C were significantly lower than those at 25 °C ( $P < 0,05$ ).

A similar pattern was recorded for nitrogenase activity over the same range of temperatures and light intensities (Figure 45). The major difference was the decline in nitrogenase activity at temperatures greater than 25 °C. Above 40 °C nitrogenase activity was zero.

At temperatures up to and including 35 °C, the *A. filiculoides* plants remained dark yellowish-green. However, at 40 °C the plants were reddish-

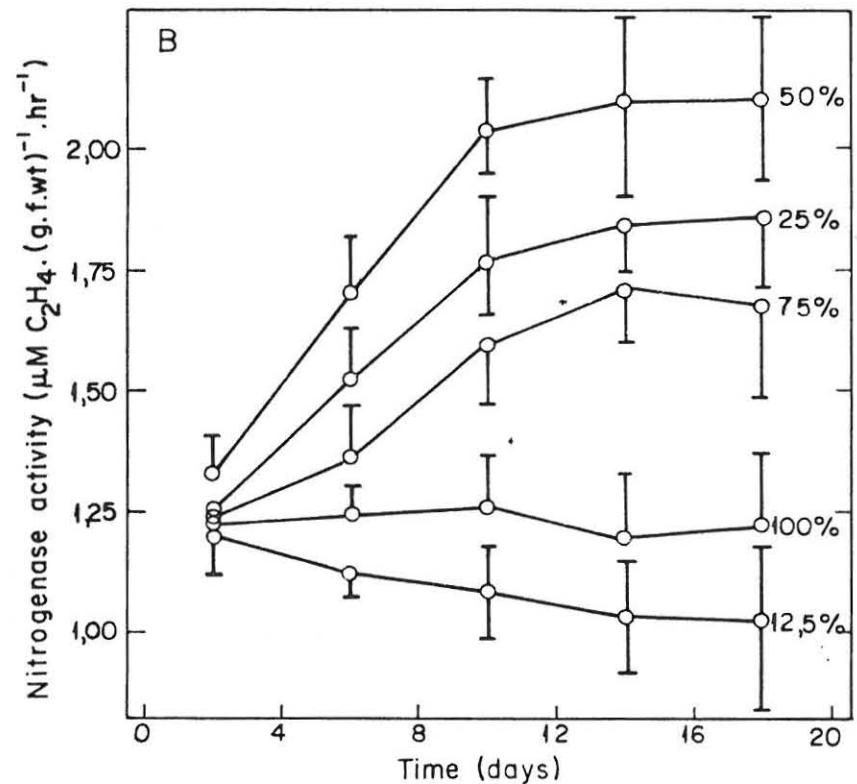
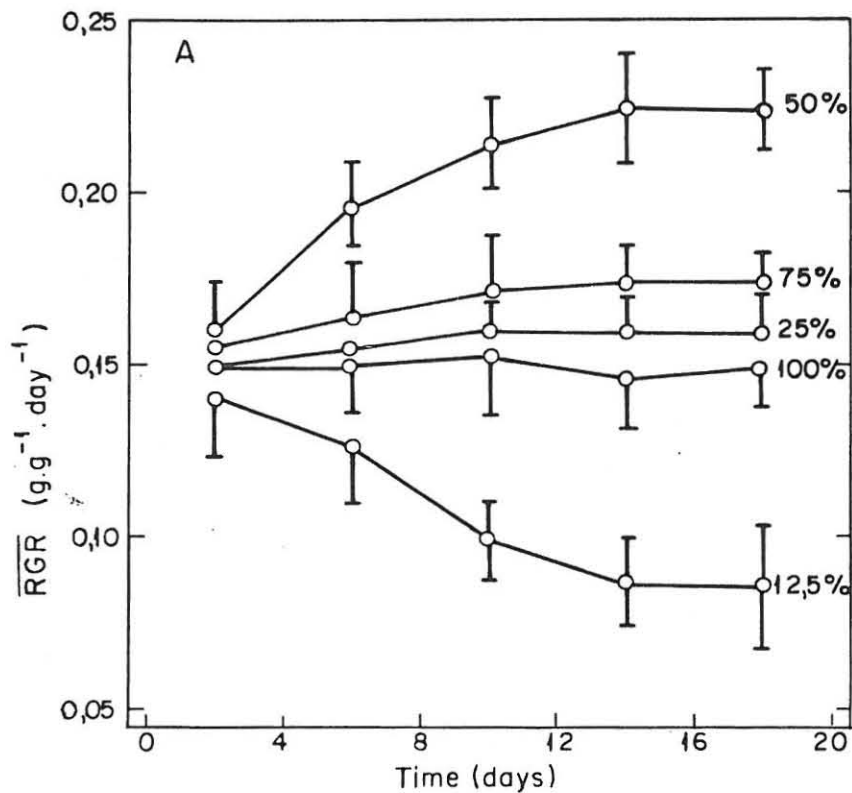


FIG. 43 Time-course experiment to show effects of different light intensities on (A) mean relative growth rate ( $\bar{RGR}$ ), and (B) nitrogenase activity of *Azolla filiculoides* plants in the field. (Field trial carried out from 27 January 1973 to 15 February 1973; mean pH 9.1; mean midday light intensity 88 kLux; maximum temperature : 30.8°C ; minimum temperature : 23.2°C). [Vertical bars indicate two standard errors].

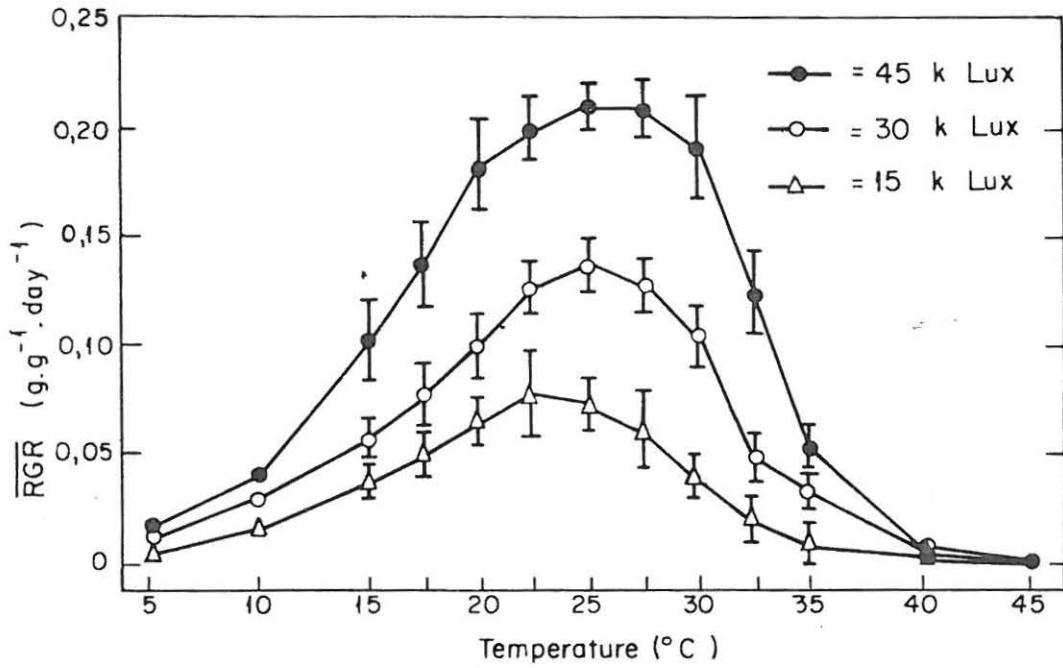


FIG. 44 *The effect of temperature on the mean relative growth rate ( $\overline{RGR}$ ) of *Azolla filiculoides* at different light intensities (14 hour day; pH 8,0). [Vertical bars indicate two standard errors].*

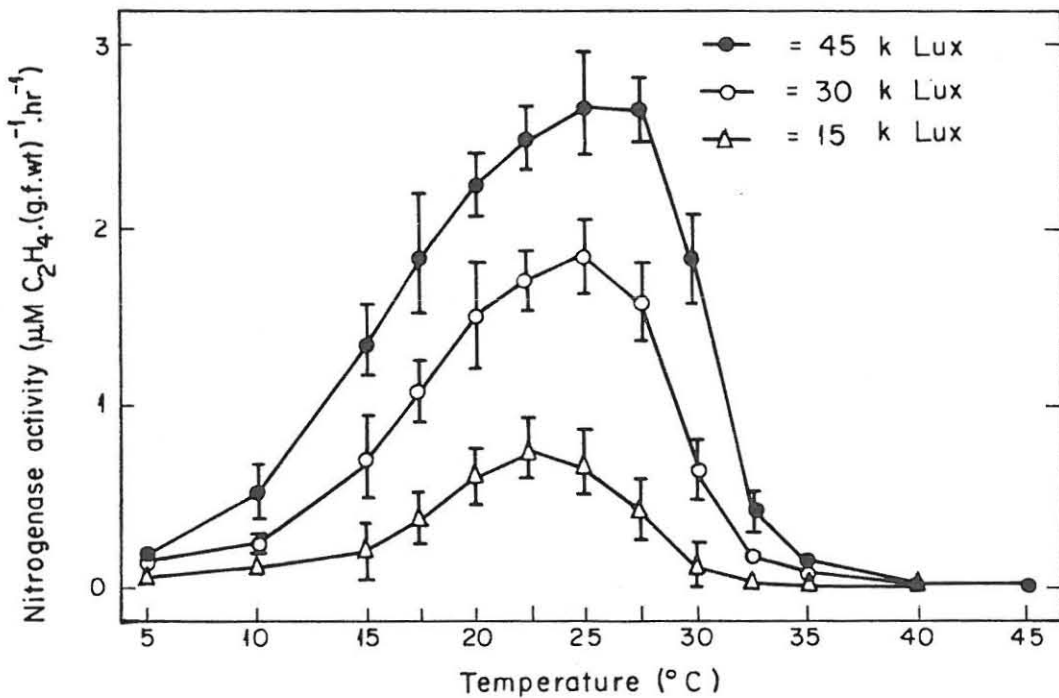


FIG. 45 *The effect of temperature on the nitrogenase activity at different light intensities (14 hour day; pH 8,0). [Vertical bars indicate two standard errors].*

brown and at 45 °C the plants were chlorotic and many (but not all) appeared bleached.

(c) Diel temperature variations

Under field conditions, temperatures are seldom constant for any length of time. Therefore, the effects of diel temperature variations at different photoperiods and light intensities were investigated. These results are shown in Tables 8 and 9.

With increasing light intensity from 5 to 60 kLux,  $\overline{\text{RGR}}$  values of *A. filiculoides* increased progressively as temperature increased from 10/5 °C to 30/20 °C at both photoperiods tested (Table 8). At higher light intensities (75 kLux)  $\overline{\text{RGR}}$  decreased. Maximum  $\overline{\text{RGR}}$  values were recorded at 35/25 °C in the longer photoperiod (14L/10D), while  $\overline{\text{RGR}}$  at this temperature was lowered in the short photoperiod treatment (10L/14D). At both photoperiods,  $\overline{\text{RGR}}$  was lowest at the 40/30 °C treatment. Under the short photoperiod treatment, the biomass doubling time decreased from 9,2 days at 10/5 °C and 5 kLux to 3,4 days at 30/20 °C and 60 kLux. Under the long photoperiod treatment, biomass doubling time decreased from 6,9 days at 10/5 °C and 5 kLux to 2,3 days at 35/25 °C and 60 kLux. *Azolla filiculoides* grew best when temperatures ranged between 25/15 °C and 35/25 °C and light intensities were between 30 and 60 kLux at a 14L/10D photoperiod.

Differences in nitrogenase activity due to changes in light intensity were smaller than those due to changes in temperature (Table 9). At low temperatures (10/5 °C) the highest nitrogenase activity was recorded at low light intensities (5 to 15 kLux). The maximum nitrogenase activity occurred at 30 kLux over the temperature range 15/5 °C to 35/25 °C under both photoperiods. Nitrogenase activity decreased at light intensities above 30 kLux and at a temperature regime of 40/30 °C was 5 % of the rate at 35/25 °C (Table 9). At all light intensities and temperature regimes, nitrogenase activity increased by 28 to 35 % as photoperiod was increased from 10L/14D to 14L/10D.

All *A. filiculoides* plants turned brownish-red in a temperature of 40/30 °C, while those plants grown at lower temperatures remained green.

TABLE 8 - Mean relative growth rate ( $\overline{\text{RGR}}$ ) of *Azolla filiculoides* ( $\text{g.g.}^{-1}\text{day}^{-1}$ ) under different photoperiods, light intensities and diel temperature regimes ( $\pm$  standard deviation,  $n = 8$  for each treatment)

Photoperiod	Temperature ( $^{\circ}\text{C}$ ) (day/night)	Light Intensity (kLux)				
		5	15	30	60	75
10L/14D	10/5	0,075 $\pm$ 0,007	0,062 $\pm$ 0,011	0,037 $\pm$ 0,011	0,021 $\pm$ 0,009	0,011 $\pm$ 0,004
	15/5	0,089 $\pm$ 0,014	0,097 $\pm$ 0,009	0,080 $\pm$ 0,010	0,068 $\pm$ 0,007	0,043 $\pm$ 0,009
	20/10	0,120 $\pm$ 0,017	0,132 $\pm$ 0,014	0,141 $\pm$ 0,020	0,150 $\pm$ 0,016	0,103 $\pm$ 0,015
	25/15	0,136 $\pm$ 0,021	0,154 $\pm$ 0,021	0,173 $\pm$ 0,019	0,192 $\pm$ 0,028	0,147 $\pm$ 0,027
	30/20	0,142 $\pm$ 0,019	0,159 $\pm$ 0,013	0,171 $\pm$ 0,021	0,204 $\pm$ 0,019	0,156 $\pm$ 0,019
	35/25	0,109 $\pm$ 0,023	0,143 $\pm$ 0,017	0,149 $\pm$ 0,013	0,203 $\pm$ 0,029	0,158 $\pm$ 0,026
	40/30	0,014 $\pm$ 0,006	0,031 $\pm$ 0,010	0,044 $\pm$ 0,009	0,052 $\pm$ 0,008	0,027 $\pm$ 0,011
14L/10D	10/5	0,101 $\pm$ 0,012	0,089 $\pm$ 0,011	0,056 $\pm$ 0,014	0,041 $\pm$ 0,009	0,022 $\pm$ 0,007
	15/5	0,124 $\pm$ 0,019	0,127 $\pm$ 0,019	0,105 $\pm$ 0,009	0,099 $\pm$ 0,012	0,072 $\pm$ 0,009
	20/10	0,155 $\pm$ 0,013	0,163 $\pm$ 0,012	0,169 $\pm$ 0,028	0,173 $\pm$ 0,025	0,127 $\pm$ 0,026
	25/15	0,186 $\pm$ 0,027	0,214 $\pm$ 0,023	0,286 $\pm$ 0,017	0,254 $\pm$ 0,028	0,179 $\pm$ 0,029
	30/20	0,193 $\pm$ 0,025	0,221 $\pm$ 0,029	0,279 $\pm$ 0,021	0,286 $\pm$ 0,024	0,212 $\pm$ 0,031
	35/25	0,126 $\pm$ 0,014	0,194 $\pm$ 0,028	0,263 $\pm$ 0,028	0,298 $\pm$ 0,031	0,217 $\pm$ 0,026
	40/30	0,025 $\pm$ 0,009	0,063 $\pm$ 0,017	0,089 $\pm$ 0,014	0,092 $\pm$ 0,009	0,042 $\pm$ 0,009

TABLE 9 - Nitrogenase activity of *Azolla filiculoides* (nmol. C<sub>2</sub>H<sub>4</sub> produced (g.f.wt)<sup>-1</sup>.hr<sup>-1</sup>) under different photoperiods, light intensities and diel temperature regimes (+ standard deviation, n = 4 for each treatment)

Photoperiod	Temperature (°C) (day/night)	Light Intensity (kLux)				
		5	15	30	60	75
10L/14D	10/5	83 ± 9	74 ± 8	68 ± 9	47 ± 9	36 ± 8
	15/5	256 ± 16	315 ± 28	403 ± 28	202 ± 23	147 ± 15
	20/10	468 ± 27	577 ± 27	1071 ± 52	839 ± 49	391 ± 29
	25/15	773 ± 31	902 ± 59	1704 ± 65	1583 ± 80	595 ± 36
	30/20	839 ± 39	1151 ± 61	1987 ± 76	1771 ± 102	982 ± 79
	35/25	597 ± 23	822 ± 28	1425 ± 53	1224 ± 67	540 ± 47
	40/30	41 ± 7	60 ± 10	76 ± 8	63 ± 9	27 ± 7
14L/10D	10/5	125 ± 16	193 ± 13	107 ± 18	70 ± 9	63 ± 12
	15/5	324 ± 23	496 ± 14	522 ± 31	407 ± 42	175 ± 16
	20/10	797 ± 39	902 ± 61	1483 ± 96	1271 ± 92	545 ± 39
	25/15	1013 ± 51	1273 ± 101	2122 ± 87	1993 ± 89	927 ± 38
	30/20	1195 ± 114	1670 ± 95	2544 ± 122	2315 ± 130	1546 ± 109
	35/25	806 ± 16	1112 ± 43	1780 ± 42	1522 ± 76	1014 ± 37
	40/30	52 ± 8	79 ± 11	101 ± 12	90 ± 11	41 ± 8

(d) pH

The influence of pH on the  $\overline{\text{RGR}}$  of *A. filiculoides* was investigated over the pH range 3 to 10 at four different light intensities. In nitrogen-deficient media, there was no indication of an interaction between light and pH (Figure 46).  $\overline{\text{RGR}}$  values were approximately equal over the pH range 5 to 8 for all light intensities used. However, both above and below this pH range,  $\overline{\text{RGR}}$  declined and was zero at pH 3 and pH 10. At all pH levels,  $\overline{\text{RGR}}$  increased with increasing light intensity between 5 and 60 kLux, but decreased at higher light intensities (75 kLux). The maximum  $\overline{\text{RGR}}$  at 60 kLux (0,24) was equivalent to a doubling time of between 2,8 and 3,0 days.

The variation in nitrogenase activity with changing pH and light intensity was similar to that recorded for the  $\overline{\text{RGR}}$  of *A. filiculoides* (Figure 47). Similar, high nitrogenase activities were recorded in the pH range 5 to 8 with zero nitrogenase activity at pH 3 and pH 10 for all light intensities. Nitrogenase activity increased with increasing light intensity from 5 to 60 kLux and decreased at 75 kLux.

The pH of the culture medium was also shown significantly ( $P < 0,001$ ) to affect the degree of tolerance to low temperatures shown by *A. filiculoides* (Figure 48). The number of viable plants dropped with decreasing pH and increased exposure times to 5 °C. At the basic pH values of 8 and 9, the number of viable *A. filiculoides* plants decreased slightly after 30 days exposure to 5 °C. At a pH of 4, less than 10 % of the *A. filiculoides* plants survived 30 days exposure to 5 °C.

(e) Photoperiod

The  $\overline{\text{RGR}}$  and nitrogenase activity of *A. filiculoides* increased with increasing photoperiod (Figure 49). Plants grown in continuous darkness died within 12 days. While the differences in  $\overline{\text{RGR}}$  at 12, 16, 20 and 24 hour photoperiods were not significantly ( $P > 0,05$ ) different from each other, 20 and 24 hour photoperiods induced significantly ( $P < 0,01$ ) higher  $\overline{\text{RGR}}$  values than a 12 hour photoperiod. Despite evidence of a decrease in nitrogenase activity at the 24 hour photoperiod, nitrogenase activities in photoperiods of 16, 20 and 24 hours were significantly ( $P < 0,05$ ) higher than those in a 12 hour photoperiod.

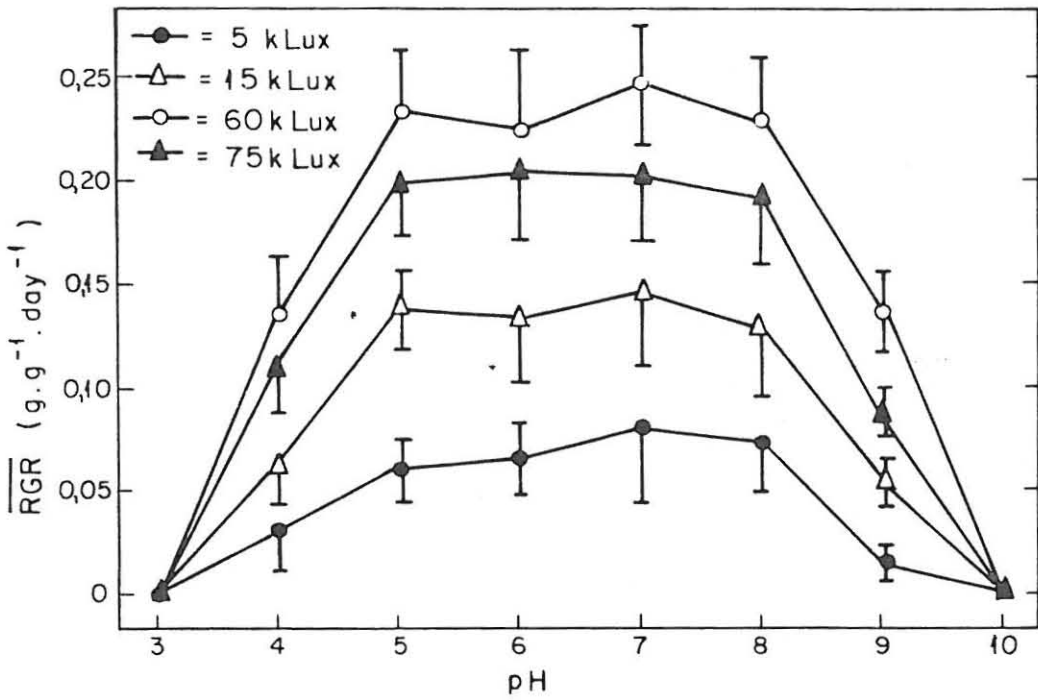


FIG. 46 The effect of different culture pH on the mean relative growth rate ( $\overline{RGR}$ ) of *Azolla filiculoides* at four light intensities (16 hour day; 27°C; N-free medium). [Vertical bars indicate two standard errors].

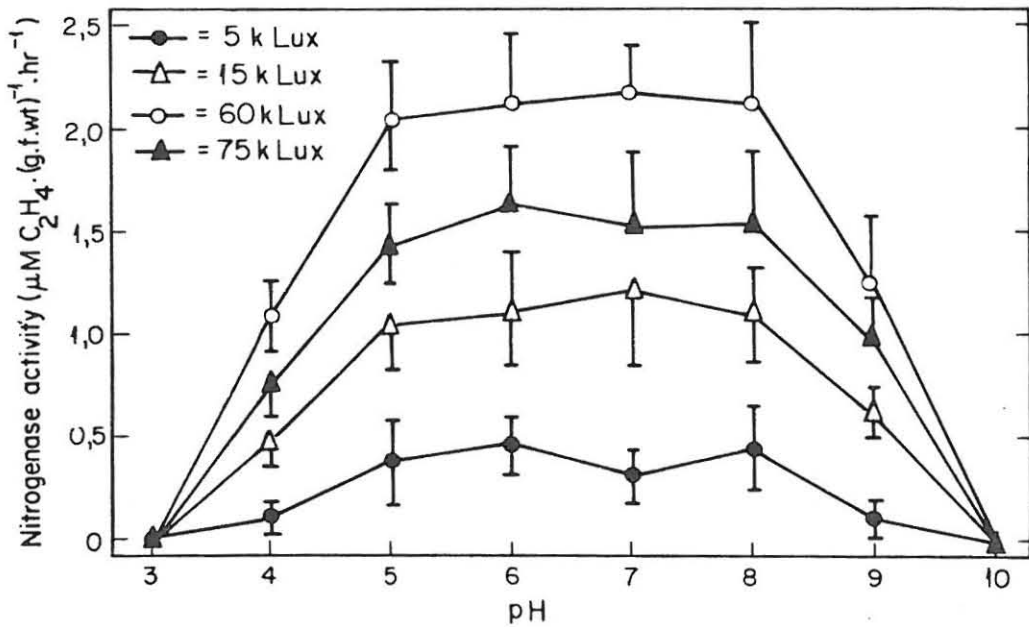


FIG. 47 The effect of different culture pH on the nitrogenase activity of *Azolla filiculoides* at four light intensities (16 hour day; 27°C; N-free medium). [Vertical bars indicate two standard errors].

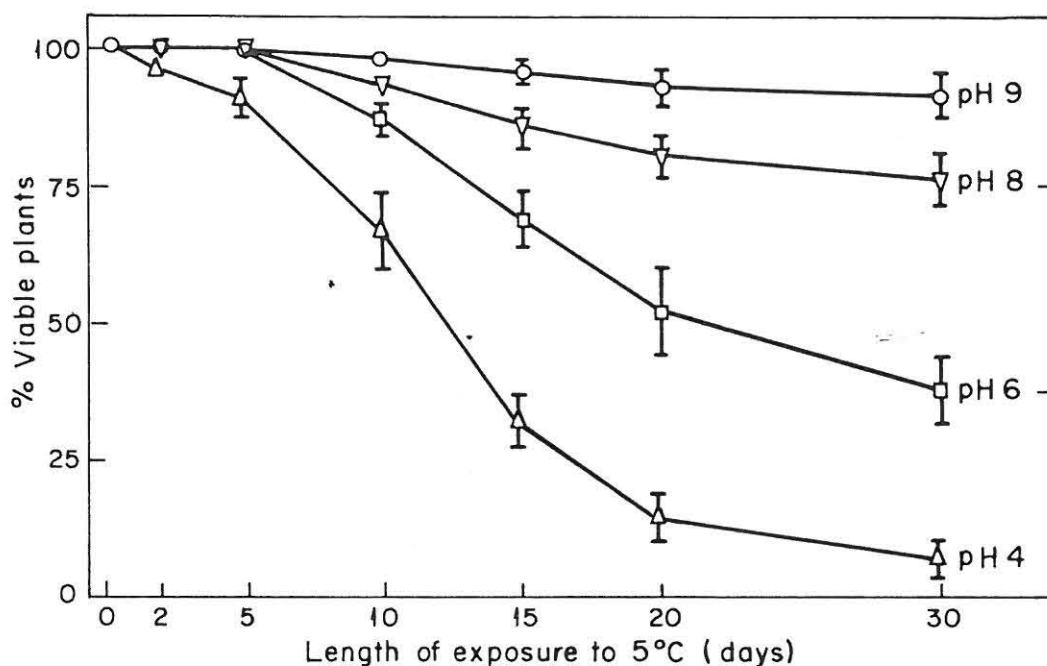


FIG. 48 *The survival (% viable plants) of large (20 mm diameter) plants of Azolla filiculoides growing at different pHs, after varying lengths of exposure to 5°C. (12 hour day; 60 kLux). [Vertical bars indicate two standard errors].*

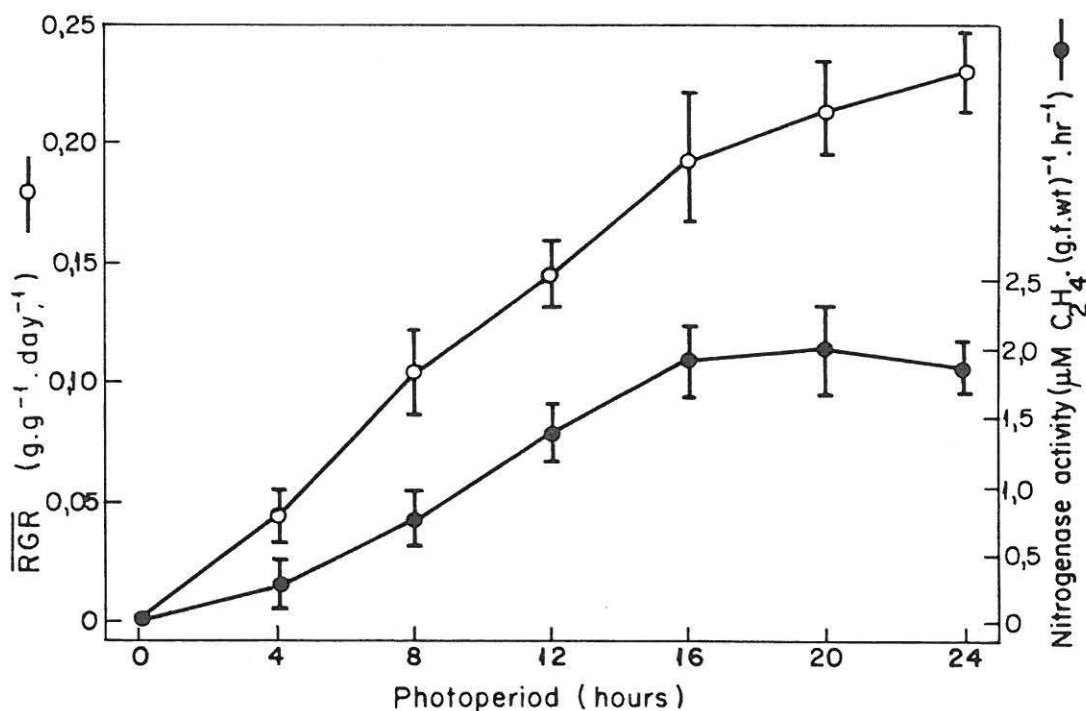


FIG. 49 *The effect of increasing photoperiod on the mean relative growth rate (RGR) and nitrogenase activity of Azolla filiculoides (60 kLux; 27°C; pH 8,0). [Vertical bars indicate two standard errors].*

(f) Light quality

Light quality markedly affected both  $\overline{\text{RGR}}$  and nitrogenase activity in *A. filiculoides* (Table 10). Both  $\overline{\text{RGR}}$  and nitrogenase activity in green light, though only 25 % of the control (white light), were still considerably higher than values for both red and blue light. Values of  $\overline{\text{RGR}}$  and nitrogenase activity increased with increasing light intensity in all treatments.

(g) Salinity and osmotic stress

The ability of *A. filiculoides* to grow on moist mud banks in field situations indicated some form of tolerance to osmotic and/or salinity stresses. Both of these aspects were separately examined in laboratory studies.

TABLE 10 - The effect of light quality upon (A) mean relative growth rate (RGR) ( $\text{g.g}^{-1}.\text{day}^{-1}$ ), and (B) nitrogenase activity ( $\text{nmol.C}_2\text{H}_4$  produced  $(\text{g.f.wt})^{-1}.\text{hr}^{-1}$ ) in *Azolla filiculoides* (values are given  $\pm$  standard deviation; n = 8 for all treatments)

Filter transmission maxima (nm)	Light intensity (kLux)	
	15	30
<u>A. Mean relative growth rate</u>		
Blue (450)	0,005 $\pm$ 0,002 a	0,007 $\pm$ 0,003 a
Green (530)	0,048 $\pm$ 0,011 b	0,065 $\pm$ 0,017 b
Red (655)	0,013 $\pm$ 0,004 a	0,017 $\pm$ 0,005 a
White (400-700)	0,186 $\pm$ 0,014 c	0,253 $\pm$ 0,031 c
<u>B. Nitrogenase activity</u>		
Blue (450)	11 $\pm$ 6 a	15 $\pm$ 8 a
Green (530)	572 $\pm$ 30 c	697 $\pm$ 42 c
Red (655)	31 $\pm$ 8 b	46 $\pm$ 11 b
White (400-700)	1349 $\pm$ 46 d	2488 $\pm$ 97 d

(Means within columns followed by similar letters are not significantly different at  $p = 0,05$ )

An increase in salinity up to 2 ‰ (NaCl concentration = 2 000  $\text{mg.l}^{-1}$ ) had no significant ( $P \gg 0,05$ ) effect on either  $\overline{\text{RGR}}$  or nitrogenase activity

in *A. filiculoides* (Figure 50). However, at salinities above 2 ‰,  $\overline{\text{RGR}}$  declined, reaching zero at 12 ‰. Nitrogenase activity remained more or less constant with increasing salinity up to 8 ‰ whereafter it declined to a minimum at 12 ‰. At a salinity of 12 ‰, the *A. filiculoides* plants appeared shriveled and chlorotic after three weeks, and therefore could not be acclimatized.

Osmotic stress due to polyethylene glycol (PEG) 6000 in the medium resulted in a decline in the  $\overline{\text{RGR}}$  of *A. filiculoides* (Figure 51). At an osmotic stress of -5 bars the  $\overline{\text{RGR}}$  was zero. Similarly, after an initial slight stimulus at -1 bars of stress, nitrogenase activity also declined to zero at -6 bars of stress. Since a salinity of 12 ‰ is equivalent to an osmotic stress of -5,05 bars, salinity and osmotic stress appeared to have identical effects on *A. filiculoides*  $\overline{\text{RGR}}$  values. However, the stress due to an osmoticum such as PEG 6000 and that due to NaCl did not produce the same effects on nitrogenase activity. A salinity of 8 ‰ (-3,37 bars) caused no appreciable loss in nitrogenase activity, while the equivalent stress caused by PEG 6000 resulted in a 70 % loss in activity. At all levels of osmotic stress induced by the addition of PEG 6000, the *A. filiculoides* plants remained green and viable after three weeks, though fronds appeared slightly flaccid at -10 bars of stress.

#### (h) Physical disturbance

Field observations showed that wind and wave action rapidly broke up mats of *A. filiculoides* and fragmented the individual fronds. The importance of physical disturbance and frond damage was therefore investigated in the laboratory.

Increasing levels of physical disturbance, in the form of agitation on a Bühler rotary shaker, fragmented the delicate *A. filiculoides* fronds (Table 11). At a shaking rate of 125 revolutions per minute and a light intensity of 15 kLux, the resulting fragments of *A. filiculoides* had a mean diameter of 2 mm. This caused an 84 % drop in  $\overline{\text{RGR}}$  and an 89 % drop in nitrogenase activity.

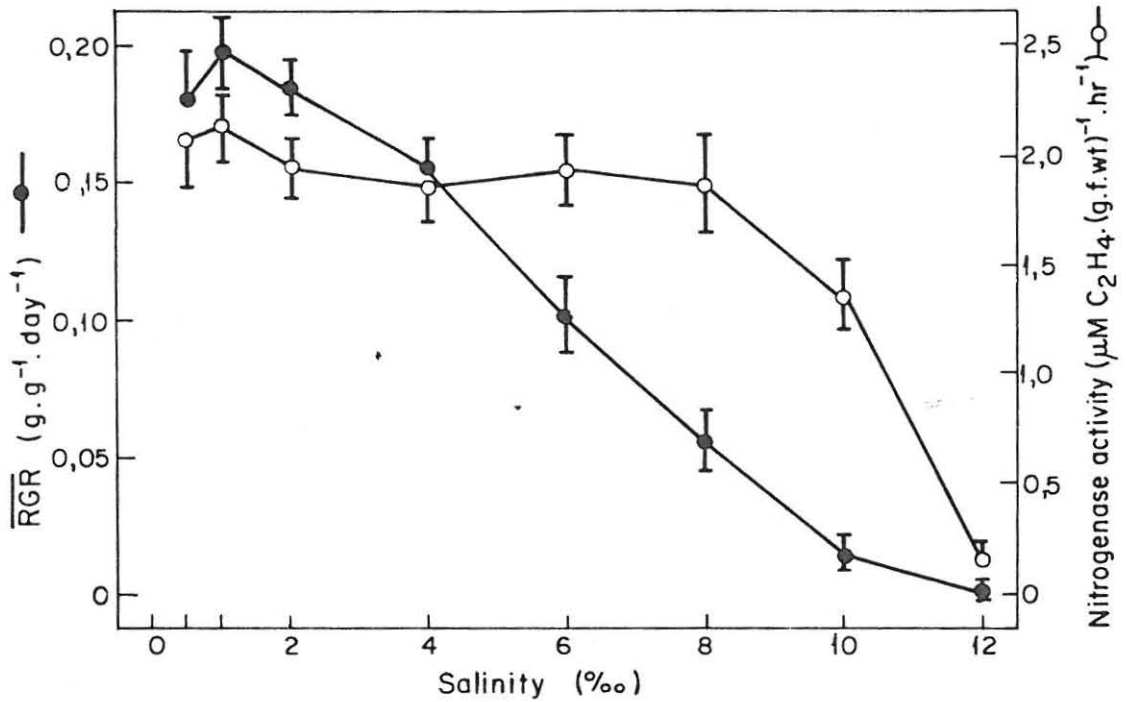


FIG. 50 *The effects of increasing salinity on mean relative growth rate ( $\overline{RGR}$ ) and nitrogenase activity of Azolla filiculoides (Basal medium = 0,51‰; 16 hour day; 27°C; pH 8,0) [Vertical bars indicate two standard errors].*

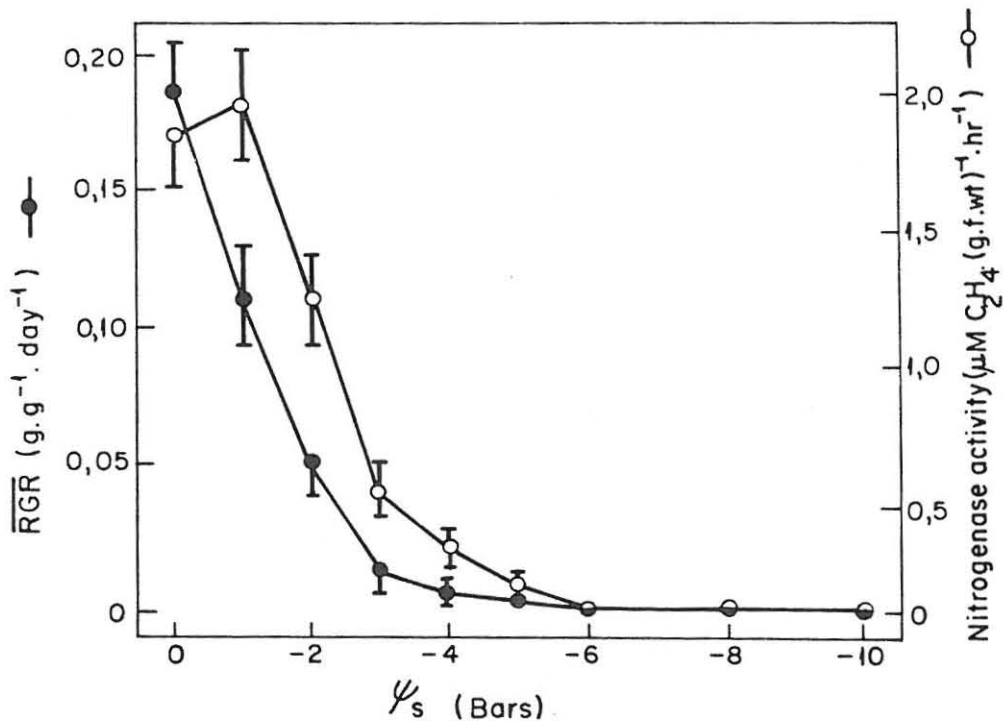


FIG. 51 *The effects of increasing osmotic stress on the mean relative growth rate ( $\overline{RGR}$ ) and nitrogenase activity of Azolla filiculoides (16 hour day; 27°C; pH 8,0). [Vertical bars indicate two standard errors].*

TABLE 11 - The effect of agitation rate on mean relative growth rate (RGR), nitrogenase activity (EPR) and resulting mean fragment size of *Azolla filiculoides* plants (values are given  $\pm$  standard deviation; n = 10 for all treatments).

Shaking rate (Revs/min)	Fragment size (mm diameter)	$\overline{\text{RGR}}$ (g.g <sup>-1</sup> .day <sup>-1</sup> )	EPR (nmol.(g.f.wt) <sup>-1</sup> .hr <sup>-1</sup> )
5	18	0,142 $\pm$ 0,029	1696 $\pm$ 98
25	15	0,124 $\pm$ 0,028	1440 $\pm$ 74
50	10	0,108 $\pm$ 0,017	1177 $\pm$ 43
75	5	0,041 $\pm$ 0,011	316 $\pm$ 24
100	3	0,035 $\pm$ 0,008	258 $\pm$ 31
125	2	0,023 $\pm$ 0,009	190 $\pm$ 36

These results were supported by measurements of the  $\overline{\text{RGR}}$  and nitrogenase activity of different sized *A. filiculoides* plants in the field (Table 12). Here, with decreasing plant size from 20 mm diameter to 2 mm diameter,  $\overline{\text{RGR}}$  decreased by 88 % and nitrogenase activity decreased by 91 %.

TABLE 12 - The mean relative growth rate ( $\overline{\text{RGR}}$ ) and nitrogenase activity (EPR) of different sizes of *Azolla filiculoides* plants in the field. (Experiment conducted at station 10, during 27-31 December 1973; values are given  $\pm$  standard deviation; n = 10 for all treatments.)

Plant size (diameter)	$\overline{\text{RGR}}$ (g.g <sup>-1</sup> .day <sup>-1</sup> )	EPR (nmol.(g.f.wt) <sup>-1</sup> .day <sup>-1</sup> )
2 mm	0,019 $\pm$ 0,007	155 $\pm$ 43
5 mm	0,047 $\pm$ 0,009	269 $\pm$ 32
10 mm	0,117 $\pm$ 0,013	1047 $\pm$ 61
20 mm	0,165 $\pm$ 0,039	1798 $\pm$ 93

In order to relate these field and laboratory results to environmental conditions and to investigate the significance of plant size, a range of different-sized *A. filiculoides* plants were subjected to increasing lengths of exposure to 75 kLux (equivalent to approximately 3/4 of full sunlight). The smallest fragments of *A. filiculoides* (2 mm diameter) had a very low tolerance to high light intensities (Figure 52), declining in viability by over 95 % within 20 days. Larger plants were more tolerant of high light

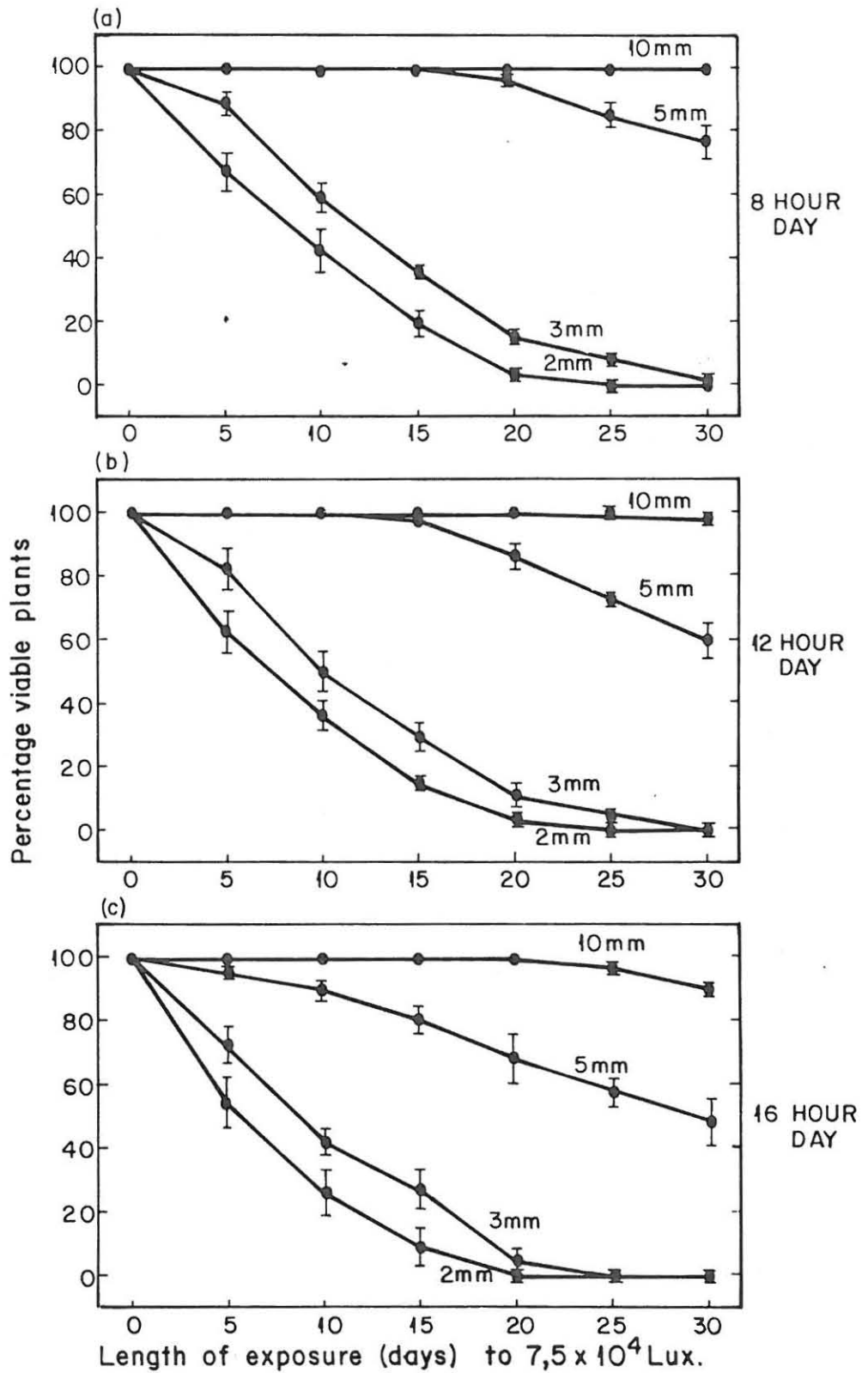


FIG.52 *The degree of tolerance to high light intensities shown by four sizes of Azolla filiculoides plants, grown in three daylength regimes. (pH8,0; 27,0°C). [Vertical bars indicate two standard errors].*

intensities. In all plant size-classes, increased photoperiod from 8L/16D to 16L/8D caused a decrease in tolerance to high light intensity.

### 5.3.3 The effects of mat formation on growth and nitrogenase activity

Observations and measurements in the field indicated that the formation of multi-layered mats greatly reduced both  $\overline{\text{RGR}}$  and nitrogenase activity in *A. filiculoides*. Within such a multi-layered mat, definite zones could be distinguished. In the lowest portion, the plants were waterlogged and senescent, bearing numerous easily-detached sporocarps. The middle layer of the mat contained rather large actively-growing plants while the plants in the top layer were mostly smaller, very slow-growing and bright orange-red in colour. In quiet undisturbed sites it was observed that mats of *A. filiculoides* would persist throughout summer whereas in open sites, the mat would be disrupted by wind and wave action, often with large portions becoming stranded on the bank. At the cessation of wind and/or wave action, the remaining plants would soon drift out over the water surface to reform the mat.

The changes in  $\overline{\text{RGR}}$  and nitrogenase activity with mat formation were examined in the field at a riverine site (station 10). In order to determine the quantity of ethylene produced per unit area, the standard incubation procedure for the acetylene reduction assay was modified. The incubation chamber consisted of a one litre capacity narrow-necked clear glass reagent bottle with its base removed. In the field, the bottle was gently placed over a portion of the *A. filiculoides* mat and then pressed through the mat with a rotating action to isolate a slice of the mat. The open base of the bottle was then sealed underwater with a wide cork shive greased with petroleum jelly to waterproof it. The height of the bottle was then adjusted by raising or lowering it, to allow an air volume of 250 ml and the bottle neck sealed with a Suba-Seal serum stopper. A single injection of 25 ml of acid-scrubbed acetylene initiated the assay, and the gas plus air phase in the bottle was mixed by repeated pumping with the syringe. When released, the bottle floated freely and could move with the *A. filiculoides* mat. The shading effect of the serum stopper was unavoidable, but must have been small since the area of the stopper was only 5 % of the area of the *A. filiculoides* mat trapped within the bottle. Two 50  $\mu\text{l}$  gas samples were removed for analysis after 20 and 40 minutes of

the incubation respectively and analysed for ethylene content as described earlier (Section 5.3.1.1). After each incubation the mat sample within each bottle was blotted dry, weighed, dried at 105 °C for 48 hours, re-weighed and analysed for nitrogen content. Nitrogenase activity was expressed as the rate of ethylene production per unit plant biomass. Because the method was so time-consuming, only 4 replicates were used for this experimental series. The study was carried out at approximately fortnightly intervals from July 1973 to January 1974. All incubations were conducted between 11h00 and 13h00, the period of maximum daily nitrogenase activity.

Growth rates ( $\overline{\text{RGR}}$ ) were measured over a period of 7 days at monthly intervals, using the procedures described in Section 2.5.2.2.

In early July, nitrogenase activity and plant nitrogen content were at a minimum (Figure 53). Biomass and nitrogenase activity increased slowly at first and then rapidly from early September to late October. Biomass continued to increase until late December whereas nitrogenase activity decreased from November onwards. With the onset of the annual floods which flushed the system in early January, biomass was reduced to a minimum and nitrogenase activity was further depressed. Plant nitrogen content increased gradually from July to early September, but during September and October, plant nitrogen content rapidly increased to 6 %. This was followed by a gradual decline in nitrogen content to the end of January. On an areal basis, the total nitrogen content of the *A. filiculoides* mat increased from 1,36 g.m<sup>-2</sup> in July to 6,85 g.m<sup>-2</sup> in December, a five-fold increase. The major portion of this increase occurred during September and October. After the annual floods in January, the total nitrogen content of the *A. filiculoides* mat dropped to 0,51 g.m<sup>-2</sup>, mainly due to the loss in plant biomass.

At the start of this field experiment, the *A. filiculoides* mat consisted of a monolayer completely covering the water surface (Figure 53). As biomass per unit area increased from 35 to 50 g.m<sup>-2</sup>, the individual fronds became larger, and curled due to the crowding effect. The fronds overlapped each other and became compressed as biomass increased to 70 g.m<sup>-2</sup>. Further increases in biomass to 90 g.m<sup>-2</sup> caused the fronds to become squashed together, and the overlapping caused the formation of a multilayered mat 3

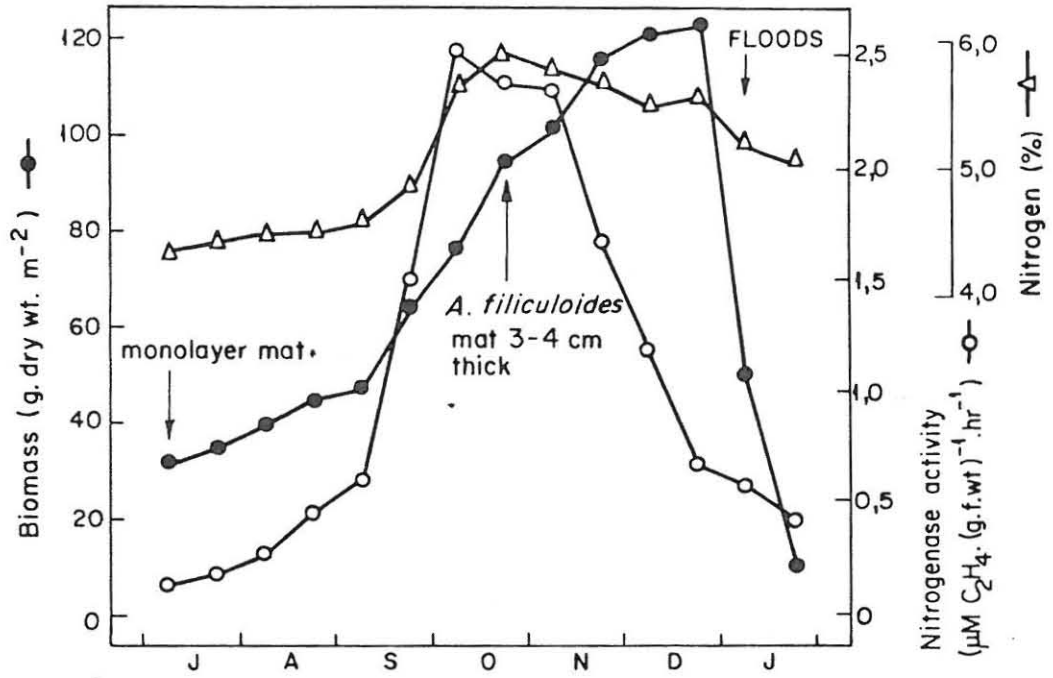


FIG.53 Fortnightly variation in biomass, nitrogenase activity and nitrogen content in *Azolla filiculoides* during mat formation at station 10 from July 1973 to January 1974.

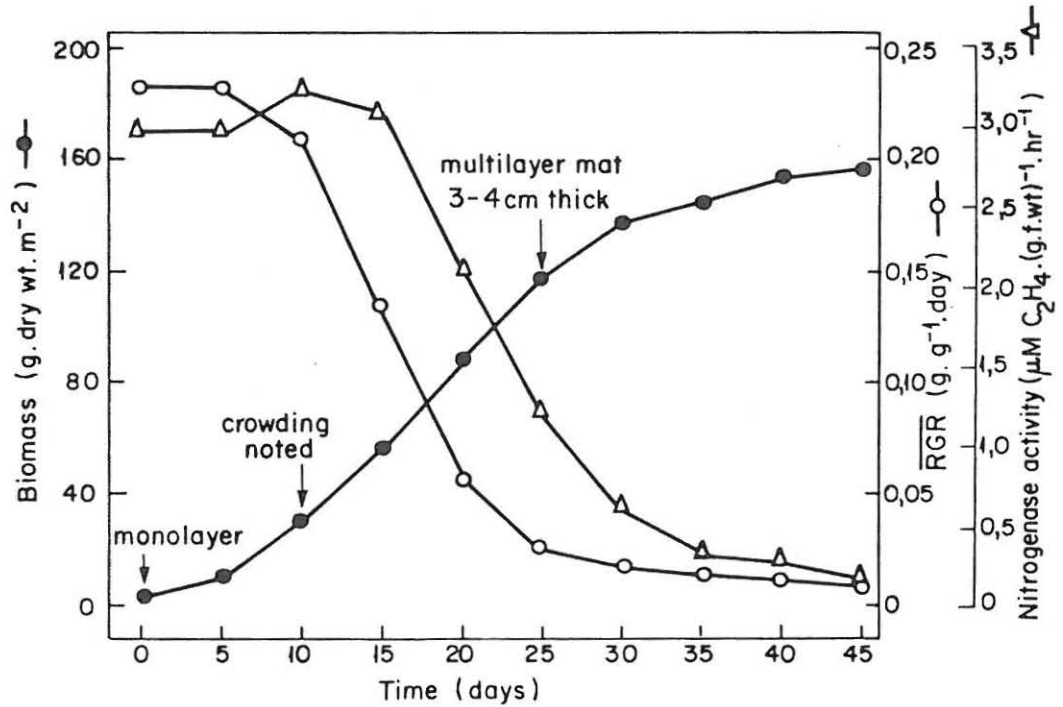


FIG.54 The changes in biomass, mean relative growth rate ( $\overline{RGR}$ ) and nitrogenase activity in *Azolla filiculoides* with time during mat formation in the laboratory. (Plants grown in 0,21 m<sup>2</sup> asbestos trays at 27°C, pH 8,0, and 14 L / 10 D photoperiod ; 45 kLux).

to 4 cm thick. With increased biomass above  $100 \text{ g.m}^{-2}$ , the mat became thicker, reaching 5 cm in thickness by late December. During mat development, the lower plants were pressed below water level by those overlying them, becoming waterlogged and chlorotic. As these lower plants senesced and rotted they were replaced by plants from the upper layers.

Examination of stranded mats of *A. filiculoides* revealed that some plants from the middle layers within the mat were green and viable up to 3 weeks after the mat had become stranded. Plants from the upper layers were completely desiccated while those from the lower senescent layers, though not desiccated, were incapable of growth on liquid media.

The process of mat formation was also followed in the laboratory (Figure 54). In the monolayer stage,  $\overline{\text{RGR}}$  and nitrogenase activity remained more or less constant. As soon as crowding became evident (between 5 and 10 days),  $\overline{\text{RGR}}$  values started to decrease while nitrogenase activity levels were maintained up to 15 days. However, with further biomass increase, a multi-layered mat formed and both  $\overline{\text{RGR}}$  and nitrogenase activity decreased to a minimum at 45 days.

#### 5.4 DISCUSSION

Interactions between environmental variables determine the suitability of a particular habitat for colonization by *A. filiculoides*. In this investigation, a combination of field observations and controlled environment studies in the laboratory were used to demonstrate the regulatory effects of a range of environmental variables on both the  $\overline{\text{RGR}}$  and nitrogenase activity of *A. filiculoides*. These results were then used to account for the limited distribution of *A. filiculoides* in the study area. Despite the fact that both  $\overline{\text{RGR}}$  and nitrogenase activity often have similar responses to a given set of environmental conditions, for convenience, these processes are discussed separately.

##### 5.4.1 Laboratory studies

###### 5.4.1.1 Growth rates ( $\overline{\text{RGR}}$ )

One of the most important results of this investigation was the finding that *A. filiculoides* plants required at least 16 days to acclimatize to a

particular treatment. Because of this, *A. filiculoides* plants in the laboratory were routinely acclimatized for at least 3 weeks in the required conditions prior to any experiments. Most other workers have used shorter acclimatization periods of 2 to 7 days and this could have led to considerable error in their results. Short acclimatization periods (a few hours) gave particularly erratic results during this investigation.

Controlled environment studies indicated that temperature was the single most important factor regulating  $\overline{\text{RGR}}$  in *A. filiculoides*, but the effects of temperature were modified by interactions with light intensity. When a diel thermoperiod was omitted and day and night temperatures were kept constant the highest  $\overline{\text{RGR}}$  values were recorded in the range 22,5 to 27,5 °C.

Reported values for temperature optima in other species of *Azolla* have been conflicting. Nguyễn-cong-Tiêu and Nguyễn-cong-Huan (1934) and Moore (1969) considered high water temperatures to be inhibitory to the growth of *A. pinnata*, while Karamyshev (1957) stated that in Vietnam, the bulk of material died off once the mean daily air temperature rose above 22 °C. This is in contrast to the results obtained by Chinese (Lu *et al.*, 1963: *loc. cit.*), Vietnamese (Tran and Dao, 1973) and Indonesian (Brotonegoro and Abdulkadir, 1976) workers. Lu *et al.* (*loc. cit.*) and Tran and Dao (1973) reported that the most favourable temperature for growth by *A. pinnata* was between 20 °C and 30 °C. Outside this range,  $\overline{\text{RGR}}$  values decreased until the plants began to die at temperatures below 5 °C and above 45 °C. In contrast, Brotonegoro and Abdulkadir (1976) reported maximum  $\overline{\text{RGR}}$  values at 40 °C. Lumpkin and Plucknett (1980) reported that at temperatures above or below the 'optimum' temperature of 25 °C, the water content and plant nitrogen content in *A. pinnata* decreased. Holst and Yopp (1979a) reported that *A. mexicana* "grew well" at temperatures up to 30 °C when the plants were cultured under low light intensities (less than 13 kLux). However, growth was reduced at 35 °C and the fern died within 7 days when cultured at 40 °C. At 5 °C, growth was not measurable but the plants remained green and viable. The inconsistencies of many of these reports reflect inadequacies in the experimental design (e.g. Karamyshev, 1957) or failure to properly acclimatize the plants used (e.g. Brotonegoro and Abdulkadir, 1976).

The comparative studies of Peters *et al.* (1980) demonstrated that the two temperate species of *Azolla*, *A. filiculoides* and *A. caroliniana* had lower temperature optima than the two tropical species *A. mexicana* and *A. pinnata*. Examination of the data provided by Peters *et al.* (1980) indicated that despite no significant difference in *A. filiculoides*  $\overline{\text{RGR}}$  values at temperatures between 20 and 30 °C, the trend is identical to that found in this study.

A similar temperature optimum has been recorded for other free-floating hydrophytes. Ashby and Oxley (1935), Blackman (1960) and Mitchell and Tur (1975) working on *Lemna minor*, *Salvinia natans* and *Salvinia molesta* respectively, showed that growth rates were highest at 30 °C but decreased rapidly at higher temperatures.

An interesting feature of published temperature preferences in *Azolla* has been the apparently contradictory findings by south-east Asian workers who have reported maximum growth for *A. pinnata* at temperatures varying between 20 and 40 °C (Nguyễn-cong-Tiêu and Nguyễn-cong-Huan, 1934; Karamyshev, 1957; Tran and Dao, 1973; Brotonegoro and Abdulkadir, 1976; Becking, 1979). While these discrepancies may be as a result of poor experimental design, the possibility exists that these are real differences indicating the presence of morphologically similar yet physiologically distinct varieties within the species. If this is so, the same may be true for other species of *Azolla* and caution is therefore required when comparing the results obtained in this study with those reported by other workers.

Most species of *Azolla* show considerable resistance to cold though freezing of the water surface for prolonged periods can result in the death of the plants (Benedict, 1923; Talley *et al.*, 1977). Low temperatures (Benedict, 1923; Shen, 1960; Pieterse *et al.*, 1977) and exposure to strong sunlight (Benedict, 1923; Svenson, 1944; Cohn and Renlund, 1953; Moore, 1969; Olsen, 1972) have also been described as favouring red colouration in *Azolla* plants.

The improved ability of *A. filiculoides* plants to withstand low temperatures when grown in the laboratory at basic pHs up to 9,0 may increase the plant's ability to over-winter in the study area. The pH of streams and dams containing *A. filiculoides* in the Hendrik Verwoerd Dam catchment area

varies between 7,5 and 9,45. Therefore, even at the lowest pH experienced by *A. filiculoides* in the field, the plants are well able to withstand long exposures to low temperatures. Tolerance of low temperatures is shared (to a lesser degree) by the other temperate species *A. caroliniana* (Benedict, 1923). The two tropical species *A. mexicana* and *A. pinnata* show poor tolerance to low temperatures (Holst and Yopp, 1979a; Becking, 1979). This aspect suggests a definite physiological difference between the temperate and tropical species of *Azolla*.

When *A. filiculoides* plants were grown in a controlled environment with a 10° temperature differential between nocturnal and diurnal temperatures,  $\overline{\text{RGR}}$  values were higher than those recorded under constant temperatures. There was an approximately linear increase in growth between 10 °C (day)/5 °C (night) and 30/20 °C, with high  $\overline{\text{RGR}}$  values maintained up to 35/25 °C. Above these temperatures  $\overline{\text{RGR}}$  decreased. Within a given temperature regime there was an approximately linear increase in biomass with increasing light intensity and maximum  $\overline{\text{RGR}}$  values were recorded at progressively higher light intensities up to 60 kLux as temperature increased. At 75 kLux  $\overline{\text{RGR}}$  was depressed by up to 25 %.  $\overline{\text{RGR}}$  values at all temperature regimes and light intensities increased as photoperiod increased from 10L/14D to 14L/10D. Talley and Rains (1980) used a 12 hour photoperiod and obtained results intermediate between the 10 and 14 hour photoperiods used here. The close correlations between temperature and  $\overline{\text{RGR}}$  in *A. filiculoides* again suggest that there is a direct physiological response to temperature in the symbiotic association.

During this investigation, the highest  $\overline{\text{RGR}}$  values for *A. filiculoides* were recorded at a light intensity of 45 kLux and these values increased with increasing photoperiod.  $\overline{\text{RGR}}$  values remained more or less constant between 45 and 60 kLux, but declined at light intensities above 60 kLux. Talley *et al.* (1977), Talley and Rains (1980) and Peters *et al.* (1980) could find only slight or no inhibition of growth and ethylene production rates at "light intensities approaching full sunlight" (Talley and Rains, 1980) in their laboratory studies. Examination of their methodology and results indicates that they were unable to attain light intensities greater than 60 kLux in their laboratories (i.e. approximately 60 % sunlight). Thus, their observed linear response of growth to light intensity is consistent with the results presented here for light intensities below 60 kLux.

Published data regarding the response of other *Azolla* species to different light intensities are similar to those presented here for *A. filiculoides*. Chinese workers (Lu, Chen, Shen and Ge, 1963: cited in Lumpkin and Plucknett, 1980) indicated that for *A. pinnata*, the highest plant nitrogen content (4,8 %) occurred at 25 kLux, while maximum rates of growth and nitrogen fixation were recorded at 47 kLux. These workers also demonstrated that *A. pinnata* survived in a range from 3,5 to 120 kLux, though 20 to 40 kLux was preferable since the plant nitrogen content was greater. Roger and Reynaud (1979) and Kulasooriya, Hirimburegama and De Silva (1980) agreed, indicating that in Senegal and Sri Lanka respectively, *A. pinnata* showed maximum rates of growth between 30 and 40 kLux. In contrast, Holst and Yopp (1979a) reported that *A. mexicana* did not grow at light intensities below 0,75 kLux and that above 20 kLux, orange-red pigmentation developed rapidly, the plants ceased growth and entered a senescent phase.

The  $\overline{\text{RGR}}$  of *A. filiculoides* (Y) showed a positive linear relationship with photoperiod (X):

$$y = 0,0099 + 0,0104 x \quad (r^2 = 0,9767; \quad n = 7; \quad P < 0,001) \quad (15)$$

*Azolla filiculoides* doubling times decreased from 30 days at a 2L/22D photoperiod to 2,9 days under constant illumination (24L/0D). The maximum  $\overline{\text{RGR}}$  attained for *A. filiculoides* under constant illumination by Peters *et al.* (1980) was 60 % higher than that reported here, and represents a doubling time of 1,8 days. The difference between the results reported here and those of Peters *et al.* (1980) is possibly due to the use of different nitrogen-free media.

Naturally-occurring symbiotic *A. filiculoides* plants grow under diurnal and seasonal fluctuations of light quality as well as quantity. Therefore, the response of *A. filiculoides* to different light wavelengths may provide further evidence for possible habitat preferences. In full sunlight *A. filiculoides* plants receive light over the whole visible spectrum (white light) while shaded plants receive primarily green light transmitted through shading vegetation plus any scattered or reflected light. The symbiotic *Anabaena azollae* in turn receives only that green light which has been transmitted through the dorsal leaf lobe. In this study, the *Anabaena* was clearly able to grow and fix nitrogen (produce ethylene) in green

light. Both blue and red light alone, although they are the primary photosynthetic wavelengths, could only support similar low  $\overline{\text{RGR}}$  values. A doubling of the light intensity did not diminish these effects. The different  $\overline{\text{RGR}}$  values recorded under green and white light reflect a 3 to 4-fold reduction in available energy and may also indicate the lack of an enhancement effect from the red and/or blue wavelengths (Holst and Yopp, 1979b).

*Azolla filiculoides* demonstrated similar  $\overline{\text{RGR}}$  values when cultured over the pH range 4 to 9. In nitrogen-deficient media, pH values between 5 and 8 were equally effective in maintaining high  $\overline{\text{RGR}}$  values. In unbuffered media or media not replaced daily, the pH dropped by over 2 pH units in a 7-day period. No significant difference in  $\overline{\text{RGR}}$  was recorded when the culture medium was replaced daily or a buffer was incorporated to maintain pH.

Other workers have reported that *Azolla* species are capable of growth from pH 3,5 to pH 10 in nitrogen-free media, with maximum growth occurring at different points between pH 5 and pH 8 (Nickell, 1958, 1961; Le Van and Sobachkin, 1963; Olsen, 1972; Watanabe *et al.*, 1977; Holst and Yopp, 1979a; Peters *et al.*, 1980). Holst and Yopp (1979a) showed that *A. mexicana* grew well from pH 4,2 to 8,0 with a small drop in  $\overline{\text{RGR}}$  at pH 8,8. Peters *et al.* (1980) demonstrated that optimal growth of 4 *Azolla* species was maintained between pH 5,0 and 8,0 with a 50 % reduction at pH 9,0. Both of these studies were carried out using buffered media. In the other studies listed above, no details were provided as to how the pH was maintained. Chemical analysis indicated that the reduction in growth at pH 9,0 in this study might have been due to iron deficiency. This aspect is described in detail in Chapter 6 of this dissertation.

In the study area *A. filiculoides* populations seldom encounter solute concentrations in excess of  $250 \text{ mg.}\ell^{-1}$  (salinity =  $0,25 \text{ }^{\circ}/_{\text{oo}}$ ). This value was well within the salinity limits shown to have no effect on  $\overline{\text{RGR}}$  values in laboratory-grown *A. filiculoides* plants. The reported salinity tolerance of *A. pinnata* differed considerably from that reported here for *A. filiculoides*. Le Van and Sobachkin (1963) reported that high salt concentrations (e.g. full strength Knop solution containing 1 500 mg salt per litre) killed *A. pinnata* within 3 weeks. *A. pinnata* plants also died in some rice fields where the salt concentration reached  $1\ 480\text{--}1\ 500 \text{ mg } \ell^{-1}$

during the summer months. Satisfactory growth of *A. pinnata* in the laboratory was only achieved after nutrient solutions had been diluted 5-10 times (c.f. lake water in Vietnam, which contained 160-380 mg salt per litre). Tran and Dao (1973) confirmed these results and suggested that the optimal concentration of mineral nutrients should be within the range of 90-150 mg  $\ell^{-1}$ . In contrast, results obtained with two New World species of *Azolla*, *A. caroliniana* and *A. mexicana*, are contradictory. Haller, Sutton and Barlowe (1974) showed that the  $\overline{\text{RGR}}$  of *A. caroliniana* declined as salinity increased, but the plants still grew well in a sea and pond water mixture of 3 000 mg  $\ell^{-1}$  solute and were not killed by a salt content of 16 000 mg  $\ell^{-1}$ . Holst and Yopp (1979a) demonstrated that *A. mexicana* could survive in a salinity range of 556-4 000 mg  $\ell^{-1}$ . Furthermore, a salt concentration of 2 000 mg  $\ell^{-1}$  in the medium had no significant effect on  $\overline{\text{RGR}}$  while at 4 000 mg  $\ell^{-1}$ ,  $\overline{\text{RGR}}$  was only slightly reduced. In order to separate ionic and osmotic effects on the  $\overline{\text{RGR}}$  of *A. mexicana*, Holst and Yopp (1979a) employed a nutrient medium containing polyethylene glycol (PEG 6000). In their experiments,  $\overline{\text{RGR}}$  steadily decreased down to a water potential of -5 bars. No growth occurred below -5 bars, but the fern remained green and viable. Holst and Yopp (1979a) concluded that since the effect of lowered water potential due to PEG paralleled that due to salt, increased salt concentrations exerted more of an osmotic effect than an ionic effect.

The results of the present study were in agreement with the trends reported by Holst and Yopp (1979a, 1979b) for *A. mexicana* and Haller *et al.* (1974) on *A. caroliniana* solute tolerance. However, the discrepancy between reported solute tolerances of New and Old World species of *Azolla* indicates that definite physiological differences exist between the subgenera (*Eu*) *Azolla* and *Rhizosperma*.

The effects of physical disturbance and fragmentation on  $\overline{\text{RGR}}$  were clearly demonstrated in laboratory trials, where  $\overline{\text{RGR}}$  values were reduced by up to 90 % by fragmentation. The only other quantitative data on this aspect were presented by Kellar and Goldman (1979). These workers showed a similar 85-90 % decrease in  $\overline{\text{RGR}}$  in a wind-fragmented population of *A. filiculoides* var. *rubra*. Braemer (1927) noted that wind-fragmented *A. pinnata* fronds had low growth rates but provided no quantitative data to support this observation.

In this study, crowding of *A. filiculoides* plants led to decreased  $\overline{\text{RGR}}$  values. This was similar to the observed decrease in *A. mexicana*  $\overline{\text{RGR}}$  values due to crowding, reported by Holst and Yopp (1979a).

#### 5.4.1.2 Nitrogenase activity

Parallel quantification of ethylene production rates (nitrogenase activity) by gas chromatography and nitrogen fixation by Kjeldahl analysis indicated a ratio of 2,21 moles of ethylene produced to 1 mole of nitrogen fixed as  $\text{NH}_3$ . This value compares favourably with the results of other workers who used the  $^{15}\text{N}_2$ -enrichment technique with different species of *Azolla*. These workers obtained mole-ratios of: 1,6 to 2,0 (Peters, Toia and Lough, 1977), 1,6 to 3,4 (Watanabe *et al.* 1977) and 2,83 to 3,01 (Kellar and Goldman, 1979). In this study the experimentally determined value of 2,21 could therefore be used to convert ethylene production rates to nitrogen fixation rates where necessary.

Controlled environment studies indicated that temperature was the single most important factor regulating nitrogenase activity in *A. filiculoides*, though, as described earlier for  $\overline{\text{RGR}}$ , the effects of temperature were modified by interactions with light intensity. When a diel thermoperiod was omitted and day and night temperatures were kept constant, maximum nitrogenase activity was recorded in the range 22,5 to 27,5 °C. Peters *et al.* (1980) reported similar temperature optima (25 °C) for nitrogenase activity in *A. filiculoides* and *A. caroliniana*.

When *A. filiculoides* plants were grown in a controlled environment with a 10° temperature differential between diurnal and nocturnal temperatures, the values for nitrogenase activity were higher than those recorded under constant temperatures. The pattern of nitrogenase activity closely followed that shown by the  $\overline{\text{RGR}}$  values described in the previous section. The results obtained here confirm the earlier studies of Talley *et al.* (1977) and Talley and Rains (1980) who also worked with *A. filiculoides*.

In this investigation, controlled environment studies indicated that nitrogenase activity in *A. filiculoides* was saturated between 30 and 60 kLux and was slightly inhibited at 75 kLux. In contrast, Peters *et al.* (1980) found nitrogenase activity to be saturated at a photon flux density of 200  $\mu\text{Moles}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$  while Talley and Rains (1980) stated categorically

that nitrogenase activity was insensitive to light intensities greater than  $50 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$ . For comparison, these intensities are approximately equal to 12 kLux and 4 kLux respectively (Harris, 1978). The assertion by Talley and Rains (1980) must be erroneous since their tabulated data clearly indicate saturation of nitrogenase activity at  $250 \mu\text{E}\cdot\text{m}^{-2}\cdot\text{sec}^{-1}$  (= 15 kLux: Harris, 1978). The reason for the discrepancy between the reports of Peters *et al.* (1980), Talley and Rains (1980) and this study is not known, but a possible explanation might be that these investigators used light sources with widely different emission spectra. Roger and Reynaud (1979) and Kulasooriya *et al.* (1980) reported that maximum rates of nitrogenase activity in *A. pinnata* were measured at 30 and 40 kLux, respectively. These light intensities correspond well to the light intensities promoting high nitrogenase activity in this study.

Because nitrogenase activity at increasing light intensities remained constant while  $\overline{\text{RGR}}$  increased, the decrease in plant nitrogen content per unit biomass with increasing light intensities was expected. Reports of increased nitrogen content with increasing light intensity (Tran and Dao, 1973) must reflect an increase in nitrogen content of the total biomass produced rather than per unit biomass.

In this study, nitrogenase activity was saturated at a 16L/8D photoperiod causing the percentage nitrogen content to decline at greater photoperiods as  $\overline{\text{RGR}}$  increased. This is consistent with the trends reported by Peters *et al.* (1980) for *A. filiculoides*.

As discussed in the previous section on  $\overline{\text{RGR}}$ , light quality also affected nitrogenase activity in *A. filiculoides*. The changes in nitrogenase activity, as a response to different light wavelengths, closely matched the changes in  $\overline{\text{RGR}}$ . These trends support the findings of an earlier study by Holst and Yopp (1979b) on *A. mexicana*.

In nitrogen-deficient media, pH values between 5 and 8 were equally effective in maintaining *A. filiculoides* nitrogenase activity at a high level. When the culture pH was above or below this range of values, nitrogenase activity decreased. These results were virtually identical to those reported by Peters *et al.* (1980) for *A. caroliniana* and *A. filiculoides*. Similarly, Holst and Yopp (1976) reported that the optimum pH for

nitrogenase activity in *A. mexicana* was 6,0, the process operating over a pH range of 4 to 8. In contrast, Watanabe *et al.* (1977) reported that nitrogenase activity in *A. pinnata* was highest at pH 5,0 and decreased at neutral pH levels.

The ability of *A. filiculoides* to maintain high nitrogenase activity levels at salinities causing a 50 to 90 % decrease in  $\overline{\text{RGR}}$  indicated that the solute concentration exercised an osmotic effect rather than a salinity effect. This was confirmed by supplementing the normal growth medium with PEG 6000 as osmoticum. Equivalent osmotic stresses induced by NaCl and PEG 6000 had almost identical effects on  $\overline{\text{RGR}}$ . However, PEG 6000 decreased nitrogenase activity by up to 70 % below the rate caused by equivalent osmotic stresses induced by NaCl. It would appear therefore that the fern must protect the algal symbiont from osmotic effects exerted by low molecular weight solutes. The mechanisms of this process are not known but must involve the active adsorption of  $\text{Na}^+$  and  $\text{Cl}^-$  ions from the osmoticum via the fern roots. An increase in % Na content of up to 120 % plus the development of toxicity symptoms in *A. filiculoides* plants grown at increasing concentrations of NaCl, provides evidence for this hypothesis. Further indirect evidence for this hypothesis is provided by the finding of Stewart *et al.* (1975) that free-living ("unprotected") species of *Anabaena* show a sharp (85 %) reduction in nitrogen-fixing ability when exposed to a low salinity of 1,4 ‰. Holst and Yopp (1979b) reported similar osmotic effects on *A. mexicana* nitrogenase activity but could not provide an explanation for the phenomenon. In their experiment, *A. mexicana* plants grown in nutrient solutions containing PEG in varying amounts showed a significant reduction in nitrogenase activity at -2,5 bars with almost total inhibition at -10 bars of osmotic stress.

In almost all of the laboratory experiments conducted during this study, the nitrogenase activity and  $\overline{\text{RGR}}$  of *A. filiculoides* plants responded similarly to a given set of environmental conditions. Therefore, on the basis of the relatively good correlation between nitrogenase activity and  $\overline{\text{RGR}}$  ( $r^2 = 0,89$ ;  $n = 140$ ), the  $\overline{\text{RGR}}$  value provides a rough index of nitrogenase activity. Thus, although a rapid rate of growth may pose potential ecological problems, it also indicates that a significant amount of nitrogen is being assimilated.

The suitability of a particular habitat for colonization by *A. filiculoides* and the environmental conditions likely to promote high  $\overline{\text{RGR}}$  and nitrogenase activity values could be predicted from an examination of the results of controlled environment studies conducted in the laboratory. The major factors which determined habitat suitability were the hydrological regime and the degree of shelter from physical disturbance by wind and wave action. It could therefore be predicted that if a riverine or farm dam site in the study area was either too exposed to wind action or dried out periodically, it would be unsuitable for *A. filiculoides*. However, because *A. filiculoides* was found to tolerate a wide range of temperatures and light intensities in the laboratory, it seemed probable that so long as a habitat was suitable in terms of the hydrological regime and degree of shelter, *A. filiculoides* plants would be able to survive unfavourable environmental conditions. In addition, it could be predicted that *A. filiculoides*  $\overline{\text{RGR}}$  and nitrogenase activity values would be maximal in a lightly-shaded environment where midday temperatures ranged between 25 and 35 °C.

#### 5.4.2 Field studies

The presence of a standing body of water was essential for the continued existence of *A. filiculoides* sporophytes throughout an annual cycle. At those sampling sites which dried up (e.g. station 6), *A. filiculoides* plants were able to survive on moist soil in the shade for periods of up to 4 weeks after which the plants died. High light intensities and both high and low temperatures favoured the production of red colouration in *A. filiculoides*, indicating that this may be a stress response to unfavourable environmental conditions.

*Azolla filiculoides* was present every month during the period February 1971 to May 1974 at only 6 of the 12 sampling sites selected (stations 4, 7, 9, 10, 11 and 12). In many north temperate countries, natural populations of *Azolla* sporophytes (*A. caroliniana* and *A. mexicana*) have been reported to completely disappear during certain seasons (Pieterse *et al.*, 1977; Talley *et al.*, 1977; Holst and Yopp, 1979a; Talley and Rains, 1980), usually as a result of plant senescence. This sort of "catastrophic senescence" did not occur during this study, indicating a possibly more favourable environment, perhaps due to latitudinal differences.

Over the 40 month duration of the field study, *A. filiculoides* biomass at both riverine and farm dam sites was strongly influenced by the hydrological regime and climatic factors. From July to January of each year, the  $\overline{\text{RGR}}$  of *A. filiculoides* could be predicted with reasonable accuracy as a positive logarithmic function of either maximum or minimum air temperature. *Azolla filiculoides*  $\overline{\text{RGR}}$  values and nitrogenase activity were minimal and the plant nitrogen content was between 3,6 and 4,2 % of dry weight throughout July and August when average air temperatures were below 12,5 °C. Between early September and late October average air temperatures increased from 13 to 19 °C. During this same period doubling times for *A. filiculoides* biomass decreased from 25 to 4,9 days, with the nitrogen content of new fronds containing 5,9 % nitrogen on a dry weight basis, an increase of approximately 50 %, due to a four-fold increase in nitrogenase activity. From October to early January the  $\overline{\text{RGR}}$  of *A. filiculoides* continued to increase, reaching a final biomass doubling time of 3,9 days. The annual floods during January to March washed out most of the *A. filiculoides* plants from the riverine site, fragmenting and damaging the remaining plants, resulting in low values for  $\overline{\text{RGR}}$  and nitrogenase activity. This pattern of development from July to January is entirely comparable with an identical pattern demonstrated for *A. filiculoides* growing in the Sacramento Valley, California, by Talley and Rains (1980).

Despite the close similarities in  $\overline{\text{RGR}}$  values at stations 9 and 10, the variations in *A. filiculoides* biomass were quite different. This was a result of the stable hydrological conditions at station 9, where flood wash-out of plants was at a minimum, allowing a multi-layered mat of *A. filiculoides* to persist for most of the study. At station 10, the annual floods removed most of the accumulated *A. filiculoides* biomass in late summer (January to March) each year. Biomass remained low during winter, followed by a rapid increase in spring to the mid-summer maximum. Because *A. filiculoides* plants were removed from the multi-layered mat at each station and their  $\overline{\text{RGR}}$  measured in open dishes, the lack of a "crowding effect" permitted high  $\overline{\text{RGR}}$  values to develop. These measurements therefore indicate the 'potential  $\overline{\text{RGR}}$ ' since crowding, which occurs in mat formation, is known to depress *Azolla*  $\overline{\text{RGR}}$  values (Talley *et al.*, 1977; Becking, 1979; Holst and Yopp, 1979a; this study).

Nitrogenase activity was poorly correlated with all environmental parameters except water temperature at station 10. At this station, nitrogenase activity followed a cyclic seasonal pattern with maxima in spring and early summer followed by minima in late summer and winter. The erratic values for the nitrogenase activity of fronds isolated from the permanent mat at station 9 may reflect the use of partly senescent material.

Diel field studies of nitrogenase activity in mid-summer indicated that nitrogenase activity was controlled by previous light treatment as well as by temperature and light intensity. On calm bright summer days the midday nitrogenase activity was always 2 to 3 times higher than those rates measured during winter or extended periods of cloudy overcast weather in summer. The finding that approximately 50 % of the total diel nitrogenase activity occurred between 10h00 and 14h30 local time has confirmed the results of Talley *et al.* (1977). In addition, significant nitrogenase activity was always recorded during the night, particularly after a calm bright summer day, confirming the findings of Talley *et al.* (1977), Kellar and Goldman (1979) and Talley and Rains (1980). This finding is in contrast to the observation by Walmsley *et al.* (1973) that nitrogenase activity decreased to zero shortly after the onset of darkness.

Measurements of  $\overline{\text{RGR}}$  and nitrogenase activity in the field indicated that these processes could be regulated by light intensity and that nitrogenase activity was saturated at lower light intensities than  $\overline{\text{RGR}}$ . The only comparable field studies aimed at investigating the effects of light intensity, are those of Lu *et al.* (1963: *loc. cit.*), Brotonegoro and Abdulkadir (1976), Roger and Reynaud (1979), Kulasooriya *et al.* (1980) and Tung and Shen (1981), all of whom worked with *A. pinnata*. These researchers demonstrated that *A. pinnata* grew best at between 30 and 50 % of full sunlight and that growth was depressed in full sunlight.

The presence of suitable habitat, protected from wind and wave action, is essential for prolonged periods of growth and development in *A. filiculoides*. The fragile structure of *A. filiculoides* fronds makes them particularly susceptible to fragmentation by physical disturbance. In the field, the abrasive action of waves easily fragmented *A. filiculoides* fronds, damaging frond apices and causing a sharp decrease in  $\overline{\text{RGR}}$  and nitrogenase activity.

During seasonal flooding of the tributary streams of the Hendrik Verwoerd Dam, numerous mats of *A. filiculoides* plants were broken up and washed downstream into the open waters of the impoundment. Here, the plants were soon fragmented by wind and wave action and carried by surface currents onto the leeward shores of the lake. The continued abrasive action of waves on the shoreline further fragmented the *A. filiculoides* fronds, such that most fragments were less than 2 mm in diameter.

Laboratory studies showed that small fragments of *A. filiculoides* were particularly susceptible to prolonged exposure to high light intensities at all photoperiods used and died within three weeks. The shoreline of the Hendrik Verwoerd Dam consists mostly of open rocky areas with no sheltering vegetation. Therefore, any *A. filiculoides* plants washed into the lake by floods and fragmented by wave action will be unable to survive for longer than three weeks, regardless of when the flooding occurs.

Mitchell (1974, 1978) stressed the importance of a stable hydrological regime for the establishment of hydrophyte communities, particularly in rivers and impounded waters. This has been highlighted by Bond and Roberts (1978) who reported the destruction of communities of *Azolla nilotica*, *Salvinia molesta*, *Pistia stratiotes* and *Eichhornia crassipes* by wind and wave action on the impounded waters of the new Cabora Bassa Dam in Mozambique.

#### 5.4.3 The significance of mat formation

Plant density is one of the major factors regulating  $\overline{\text{RGR}}$  and nitrogenase activity in *A. filiculoides*. In undisturbed optimal conditions, *A. filiculoides* grew rapidly until the available surface area was covered. Further growth caused overlapping of fronds and the formation of a multi-layered mat. Multi-layered mats are characteristic of all *Azolla* populations growing in undisturbed locations and have been reported by many authors (e.g. Benedict, 1923; Saubert, 1949; Becking, 1976b; Kellar and Goldman, 1979; Lumpkin and Plucknett, 1980).

Within an established mat, *A. filiculoides* plants are protected from high light intensities by the overlying mat layer(s) and are partly insulated from extremes of temperature. (Field measurements showed a 3 to 5 °C

temperature differential between upper and lower mat layers.) Spores are produced only by senescent plants in the lower layers of established mats and these therefore provide a means of continuing the sexual phase of the fern's life cycle. Senescence and mineralization of the lower mat layers also ensure a nutrient supply to the upper mat layers. The reduction in nitrogenase activity of *A. filiculoides* plants in established mats is not due to nutrient limitation, but appears to be caused by an alteration in the fern's nitrogen metabolism. (This aspect is discussed in Chapter 8.)

Mat formation is therefore a key feature of *A. filiculoides* growth and contributes to the fern's ability to survive unfavourable environmental conditions. Comparisons of *A. filiculoides* growth and mat formation at a sheltered site (station 9: farm dam) and at a poorly-sheltered site (station 10: riverine) in this study have emphasized the importance of suitable habitat for mat formation. The degree of physical disturbance by wind and wave action in a particular habitat is a critical factor for successful mat formation in *A. filiculoides*, and therefore determines the success or failure of the plant to colonize that habitat.

## CHAPTER 6

### NUTRIENT REQUIREMENTS OF THE SYMBIOTIC ASSOCIATION

#### 6.1 INTRODUCTION

The growth of *Azolla* plants is dependent on an adequate supply of the macro- and micronutrients required for normal plant growth and, like most plants, *Azolla* is sensitive to changes and deficiencies in the supply of plant nutrients (Moore, 1969; Lumpkin and Plucknett, 1980). Smith (1938) considered that the lower lobes of *Azolla* leaves were the principal site of uptake of water and nutrients. However, Schaeede (1947), using dyes, demonstrated that this uptake proceeds via the roots, with the lower leaf lobes being involved only to a very minor degree.

Despite the fact that several workers have reported on the nutrient requirements of *A. caroliniana* (Cohn and Renlund, 1953; Nickell, 1958, 1961; Olsen, 1972) and *A. pinnata* (Saubert, 1949; Le Van and Sobachkin, 1963; Watanabe *et al.*, 1980; Subudhi and Singh, 1979a, b; Yatazawa *et al.*, 1980), surprisingly little information has been published on the nutrient requirements of *A. filiculoides*. Talley *et al.* (1977) reported that the growth of natural populations of *A. filiculoides* in California appeared to be limited by the availability of P and Fe. These authors also noted that foliar symptoms of P-deficiency (i.e. chlorosis) disappeared when water levels dropped, allowing the *A. filiculoides* roots to come into contact with bottom sediments. Because nitrogen fixation by the algal symbiont plays a dominant role in regulating the growth of the fern (Ashton and Walmsley, 1976), those micronutrients which have been shown to be essential for the nitrogen-fixing process in nodulated legumes and free-living blue-green algae (Fogg, 1962) should also be present in adequate supply. In this regard, Bortels (1940) and Johnson, Mayeux and Evans (1966), respectively, have shown that *A. filiculoides* required a supply of Mo and Co, though only when grown in nitrogen-deficient media, confirming that the fern required these elements specifically for nitrogen fixation. Bottomly (1920) reported that an extract from "bacterized peat" caused a 40 % increase in the yield of *A. filiculoides* over an 11-week period. However, this could equally well have been the result of micronutrients in the extract (Moore, 1969) rather than the presence of "organic growth-promoting substances".

In the Hendrik Verwoerd Dam catchment area, concentrations of the dissolved plant nutrients N, P, K, Ca, Mg, Na, Fe, Cu and Zn in streams and farm dams are generally very low and yet *A. filiculoides* is able to grow and proliferate, apparently with no ill-effects. However, it must be remembered that in an aquatic environment, nutrient availability is governed by the chemical conditions beneath the water surface, particularly the pH and oxygen status (Hutchinson, 1957). Indeed, in the natural habitat of *A. filiculoides* nutrients may be present in large quantities but, because of unsuitable pH and oxygen regimes, are not in a form available for uptake by the fern (Ashton and Walmsley, 1976). In this regard, mat formation by *A. filiculoides* can drastically alter the chemical characteristics of the underlying waters, but the effects of these changes in water chemistry on *A. filiculoides* have not previously been quantified.

The nitrogen-fixing capacity of the algal symbiont in *Azolla* can supply the nitrogen requirements of both partners in the association and allows *A. filiculoides* to flourish in waters free of combined nitrogen (Ashton and Walmsley, 1976). Because of this feature, many workers have utilized nitrogen-free culture media for their experimental studies on the nutrient requirements of other *Azolla* species. The nitrogen metabolism of the fern-alga association will be dealt with in Chapter 8 of this dissertation. The aims of this investigation were to:

- (a) quantify the nutrient requirements of *A. filiculoides* and the effects of nutrient deficiencies on growth and nitrogenase activity in *A. filiculoides*;
- (b) determine the seasonal variations in nutrient availability in the Hendrik Verwoerd Dam catchment area and their effects on *A. filiculoides*;
- (c) investigate the effects of mat formation on water chemistry and subsequent effects on *A. filiculoides* growth and nitrogenase activity.

## 6.2 MATERIALS AND METHODS

### 6.2.1 Selection of suitable culture media

The choice of a suitable inorganic culture medium for *Azolla filiculoides* was complicated by the variety of media that have been used by previous workers. Dilutions of modified Knop's, Hoagland's or Chu's formulae, as well as media designed for growth of nitrogen-fixing blue-green algae, have all been reported to support *Azolla* growth. Initially, eight media formulated by Saubert (1949), Gerlof, Fitzgerald and Skoog (1950), Allen and Arnon (1955), Nickell (1958), Clatworthy and Harper (1962), Gorham, McLachlan, Hammer and Kim (1964), Johnson *et al.* (1966) and Lahdesmaki (1968) were selected for testing as to their suitability for the culture of *A. filiculoides*. Two further media, proposed by Olsen (1972) and Peters and Mayne (1974a), were also tested at a later stage of this study.

The media varied considerably in both concentration and range of nutrient salts used, particularly sources of Fe and micronutrients. Therefore, in order to facilitate comparison, three formulations of micronutrients proposed by Allen and Arnon (1955), Johnson *et al.* (1966) and Gorham *et al.* (1964) were selected for testing and Fe was supplied as the ferric-monosodium-EDTA (Fe-Na-EDTA) salt to all media. All media were free of combined nitrogen. The macronutrient composition of the ten media tested are shown in Table 13 and the micronutrient composition of the three formulations selected are shown in Table 14. All chemicals used were analytical grade and no further purification was made. The water used in the preparation of culture solutions was first distilled and then de-ionized by passing through Amberlite MB3 ion exchange resin. Stock solutions were made up so that each solution contained 250 millimoles of the appropriate nutrient. All glassware was cleaned with 3N HCl or 3N HNO<sub>3</sub> followed by rinsing with large volumes of distilled water.

Each medium was tested in batch culture at three levels of concentration (half-, full- and double-strength) with each of the three micronutrient combinations. The pH of all culture solutions was adjusted to 6,0 with either 1N HCl or 1N NaOH.

TABLE 13 - Macronutrient composition of different media tested for growth of *A. filiculoides*

A. Concentration of nutrient salt in solution (mmoles.l <sup>-1</sup> )*										
Salt	A	B	C	D	E	F	G	H	I	J
MgSO <sub>4</sub> .7H <sub>2</sub> O	5,0	0,1	1,0	2,0	0,4	0,4	0,2	0,2	1,3	0,8
CaCl <sub>2</sub> .2H <sub>2</sub> O	2,4	0,2	0,5	5,0	1,3	0,2	0,1	1,0	-	2,0
CaSO <sub>4</sub> .2H <sub>2</sub> O	-	-	-	-	-	-	1,0	-	0,8	-
K <sub>2</sub> SO <sub>4</sub>	2,5	-	-	2,0	-	-	0,5	-	0,4	-
K <sub>2</sub> HPO <sub>4</sub>	2,4	0,1	2,0	-	-	0,1	0,1	-	-	-
KH <sub>2</sub> PO <sub>4</sub>	-	-	-	1,0	0,7	-	0,2	0,3	0,5	0,4
KCl	-	0,5	-	2,0	2,5	-	-	0,4	-	2,0
NaCl	-	-	4,0	-	-	2,0	-	-	0,4	-
Na <sub>2</sub> HPO <sub>4</sub>	-	-	-	-	-	0,1	-	-	-	-
Fe-Na-EDTA	0,07	0,07	0,07	0,07	0,07	0,07	0,07	0,07	0,07	0,07

B. Macronutrient concentration in solution (mg.l <sup>-1</sup> )										
Element	A	B	C	D	E	F	G	H	I	J
Ca	96	8	20	200	52	8	44	40	32	80
Mg	121	2,5	24	48	10	10	5	5	32	19
Na	-	-	92	-	-	51	-	-	9	-
K	382	27,5	156	274	125	8	55	27,5	51	94
P	75	3	62	31	22	6,5	9,5	9,5	15,5	12,5
S	240	6,5	32	128	13	13	54	6,5	80	26
Cl	170	31,5	177	425	181	85	7	85	14	218
Fe	4	4	4	4	4	4	4	4	4	4

\*A: Saubert (1949); B: Gerlof *et al.* (1950); C: Allen and Arnon (1955); D: Nickell (1958); E: Clatworthy and Harper (1962); F: Gorham *et al.* (1964); G: Johnson *et al.* (1966); H: Lahdesmaki (1968); I: Olsen (1972); J: Peters and Mayne (1974a)

TABLE 14 - Composition of micronutrient solutions tested for growth of *A. filiculoides*

A. Concentration of nutrient salt in ( $\mu\text{moles.l}^{-1}$ )\*

Salt	A	B	C
$\text{H}_3\text{BO}_3$	45,50	5,82	40,00
$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	9,10	1,13	7,00
$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	0,77	0,18	3,20
$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	1,04	0,05	0,08
$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0,31	0,08	0,0008
$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0,17	0,09	0,50

B. Micronutrient concentration in solution ( $\mu\text{g.l}^{-1}$ )

Element	A	B	C
B	500,5	64,0	440,0
Mn	500,5	62,2	385,0
Zn	50,0	11,7	208,0
Mo	99,8	4,8	4,7
Cu	19,8	5,1	0,05
Co	10,0	4,7	48,0

\*A: Allen and Arnon (1955);

B: Johnson *et al.* (1966);

C: Gorham *et al.* (1964)

For the analysis of growth rates, weighed samples (approximately 1,0 g) of acclimatized *A. filiculoides* plants were freed of epiphytic contaminants as described in Section 2.5.2.2, placed in 500 ml capacity plastic dishes containing the appropriate nutrient solution and reweighed at four-day intervals for 20 days. Each treatment was replicated ten times and nutrient solutions were replaced daily by siphoning. When crowding was noted, half the biomass was removed to a second container of the culture solution under test. All tests were carried out under a photoperiod of 16L/8D, at a light intensity of 45 kLux and at a temperature of 27 °C. After 20 days had elapsed, the nitrogenase activity of *A. filiculoides* plants in each treatment was assessed with the acetylene reduction assay (Section 5.3.1.1). The combination of nutrient medium and micronutrient solution giving the highest values of  $\overline{\text{RGR}}$  and nitrogenase activity was then selected for further optimization and routine laboratory use.

#### 6.2.2 Determination of optimum levels of individual nutrients

In order to formulate a nutrient solution containing optimum levels of each macronutrient, the effects of different concentrations of individual nutrients were assessed in batch cultures. This was accomplished with the optimum nutrient solution selected in 6.2.1 above. The solution was modified by adding the selected micro- or macronutrient under test at levels of 4X, 2X, 1X, 1/2X, 1/4X, 1/8X, 1/16X and 1/32X the normal recipe concentration, while maintaining the other macro- and micronutrients at a constant level. The pH of each culture solution was adjusted to 6,0 with either 1N HCl or 1N NaOH. Prior to each experiment, the *A. filiculoides* plants were grown for three weeks in the appropriate nutrient solution in order to acclimatize the plants. Weighed samples (approximately 1,0 g) of acclimatized, surface-sterilized *A. filiculoides* plants were placed in 500 ml capacity plastic dishes containing 450 ml of the appropriate nutrient solution. Each treatment was replicated ten times and again nutrient solutions were replaced daily by siphoning. Growth rates were measured over a seven-day period and nitrogenase activity was measured on the seventh day with the acetylene reduction assay (Section 5.3.1.1). All experiments were carried out under a photoperiod of 16L/8D, at a light intensity of 45 kLux and at a temperature of 27 °C.

### 6.2.3 Determination of threshold or minimum nutrient requirements

In traditional static or batch culture experiments, the amounts of nutrient supplied are generally sufficient at the beginning of an experiment but later, during rapid growth, become deficient. The optimum nutrient level is then dependent on the volume of the culture and the frequency of culture solution renewal. In order to overcome these difficulties as far as possible and to determine the minimum concentration of a nutrient required to sustain optimum growth, a continuous-flow culture is essential. An additional advantage of continuous-flow cultures is that they tend to simulate the conditions that prevail in slow-flowing riverine and stream habitats better than static or batch cultures.

Each continuous-flow culture consisted of four 500 ml capacity plastic dishes, each with an outlet hole located so as to maintain a volume of 450 ml in the dish. Culture solution was contained in a 20 l capacity reservoir, connected to the culture dishes by flexible polyvinyl chloride (PVC) tubing and flow rates were controlled by a tap. Both the reservoir and the PVC tubing were covered with aluminium foil to prevent algal growth, and the reservoir was refilled with culture solution each day. Each dish contained a weighed sample (approximately 1,0 g) of previously acclimatized, surface-sterilized *A. filiculoides* plants. Each treatment, consisting of four dishes, was replicated twice. Growth rates were measured at four-day intervals for 20 days and nitrogenase activity was measured on the twentieth day with the acetylene reduction assay (Section 5.3.1.1).

Several series of experiments were carried out, using different levels of each macronutrient at a series of flow rates. Details of nutrient concentrations and flow rates are shown in the relevant results. With the apparatus described above, flow rates from 500 ml to 5,0 l per hour could be accurately controlled.

### 6.2.4 Nutrient deficient media and ion interactions

An important aspect of the nutrition of *A. filiculoides* concerns the effects of nutrient deficiencies on growth and nitrogenase activity. Traditionally, studies on the effects of nutrient deficiencies have been carried out in

batch cultures (e.g. Bortels, 1940; Le Van and Sobachkin 1963), in which one component of the complete nutrient solution is eliminated and the resulting plant growth compared with that of control plants growing in complete nutrient solution. The ability of plants to store excess nutrients and the fact that several elements are highly mobile in plant tissue, being transported with ease from old to young tissue, can confuse the interpretation of results. Thus, the effects of nutrient deficiencies may not become apparent for some time, necessitating repeated transfers to fresh culture media. In addition, the interaction between nutrient ions in solution and in plant tissue can also obscure the effects of nutrient deficiencies (Olsen, 1972). During this investigation, both the direct effects of nutrient deficiencies and the interactions between nutrient ions in solution were studied.

Media deficient in Ca, Mg and K were prepared by eliminating each one of these components from the complete solution and replacing it with an equivalent weight of the appropriate Na salt to maintain ionic balance. Phosphate- and sulphate-deficient media were prepared by substituting an equivalent weight of the appropriate chloride salt. Micronutrient deficient media were obtained merely by eliminating the appropriate micronutrient from the culture solution. Since the micronutrient concentrations were so low, no attempt was made to maintain ionic balance. In each case, the pH of the culture medium was adjusted to 6,0 with either 1N HCl or 1N NaOH.

Cultures of *A. filiculoides* were grown in 1 l capacity cotton-wool capped Erlenmeyer flasks containing 450 ml of medium. All media and glassware were sterilized prior to the experiment by autoclaving at 15 psi for 20 minutes. Each flask was inoculated with approximately 1,0 g of surface-sterilized *A. filiculoides* plants. Every four days, the *A. filiculoides* plants were transferred to fresh sterile media. Although the *A. filiculoides* cultures were not axenic, they were handled by sterile techniques in order to minimize colonization of the cultures by undesirable algal populations. Filtered, humidified air was introduced into each flask to maintain the levels of atmospheric gases. Each treatment was replicated six times and  $\overline{\text{RGR}}$  and nitrogenase activity were measured every four days during transfer to fresh media, for a period of 32 days. When

crowding was noted, half of the biomass was transferred to a second flask of the appropriate medium.

The nutrient triangle technique of Hamner, Lyon and Hamner (1942) was employed to investigate the effects of media deficient in two nutrients as well as the interacting effects of nutrient ions at sub-optimal concentrations. The technique was originally designed by Hamner (1940) and Hamner *et al.* (1942) in nutritional studies on soybeans and tomatoes. Mitchell (1970) subsequently modified this technique to study nutrient deficiencies in *Salvinia molesta (auriculata)*. The modified version designed by Mitchell (1970) used nutrient levels approaching those in lake water, very much lower than those used by Hamner *et al.* (1942). In both of the above studies, the three anions used were  $\text{NO}_3$ ,  $\text{PO}_4$  and  $\text{SO}_4$  and, in order to maintain nitrogen-free media for *A. filiculoides* in this study, Cl was substituted for  $\text{NO}_3$ .

The preparation of nutrient stock solutions and their combination into nutrient triangles was similar to that described by Mitchell (1970). Three cation and three anion stock solutions were prepared in the concentrations shown in Table 15. Each cation solution contained a mixture of Cl,  $\text{PO}_4$  and

TABLE 15 - Ionic concentrations of nutrient stock solutions in milliequivalents per litre\*

Salt	Ionic concentrations of individual stock solutions					
	K	Ca	Mg	Cl	$\text{PO}_4$	$\text{SO}_4$
KCl	1			1		
$\text{KH}_2\text{PO}_4$	1				2	
$\text{K}_2\text{SO}_4$	1					2
$\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$		2		1		
$\text{CaHPO}_4 \cdot 2\text{H}_2\text{O}$		1			1	
$\text{CaSO}_4 \cdot 2\text{H}_2\text{O}$		1				1
$\text{MgCl}_2 \cdot 6\text{H}_2\text{O}$			2	1		
$\text{MgHPO}_4 \cdot 3\text{H}_2\text{O}$			1		1	
$\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$			1			1

\*Each final solution was supplemented with suitable levels of iron and micronutrients (6.2.1)

SO<sub>4</sub> salts of that cation and each anion solution contained the Ca, Mg and K salts of that anion. Suitable combinations of the three cation or anion stock solutions were then devised on the basis of one-eighths of the final mixture to form a nutrient triangle composed of 45 different solutions as shown in Figure 55. Thus the cation triangle contained solutions of different ionic concentrations of cations, but the same concentrations of anions, while the situation was reversed in the anion triangle. Thus, the apices of each triangle were deficient in two ions, the sides were deficient in one ion and the centre portion of each triangle was supplied with all three ions in varying proportions. Therefore, it was anticipated that the relative importance of each of the ions plus any signs of deficiencies or interactions could be obtained.

Twenty-one cation treatments and twenty-one anion treatments were selected from each of the 45 possible cation and anion treatments in order to cover the range of concentrations for each element. The positions of the twenty-one selected treatments within each of the 45 possible combinations are shown in the nutrient triangle in Figure 55.

The *A. filiculoides* plants were grown in plastic dishes containing 450 ml of nutrient solution. All solutions were adjusted to pH 6,0 with either 1N HCl or 1N NaOH and replaced daily by siphoning. Each treatment was replicated eight times and laid out in the constant environment room in a randomized pattern. Each dish was inoculated with approximately 1,0 g of *A. filiculoides* plants. Growth rates were measured at 4-day intervals for 20 days and nitrogenase activity was measured on the twentieth day using the acetylene reduction assay (Section 5.3.1.1). All experiments were carried out under a photoperiod of 16L/8D, at a light intensity of 45 kLux and a temperature of 27 °C.

#### 6.2.5 Iron availability and the effects of pH on nutrient uptake

In most aquatic plants, water pH plays an important role in regulating the rates of uptake of certain nutrients, particularly Ca, P and Fe (Sculthorpe, 1967; Olsen, 1972). The cations Ca, Fe and Mn have been shown to interact, interfering with their uptake, the degree of interaction varying with pH, both in algae (Tel-Or and Stewart, 1975) and aquatic macrophytes (Olsen, 1972; Hutchinson, 1975).

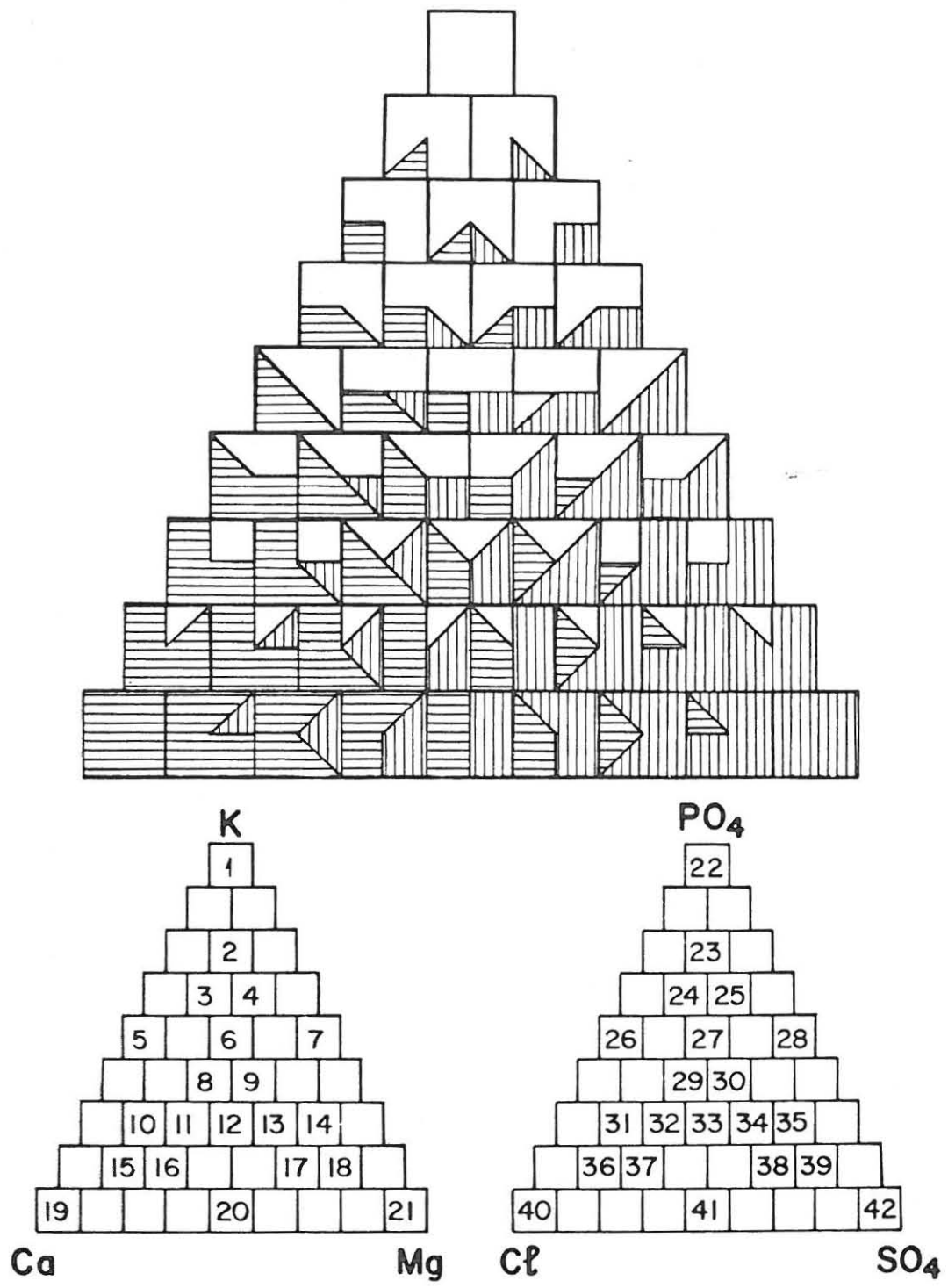


FIG. 55 Upper triangle is a diagrammatic representation of the relative proportions of stock solutions used to produce 45 possible combinations. Left, below : 21 combinations of cations selected and right below : 21 combinations of anions selected.

The influence of culture pH on nutrient interactions and availability to *A. filiculoides* was investigated in laboratory batch cultures, using the complete (nitrogen-free) medium selected in Section 6.2.1 as control. Two levels of Ca (4X and 1X the normal recipe concentration) were selected for study. Within each of these treatments, Fe was supplied either as the ferric or ferrous salt, with or without a chelating agent (citric acid). The nutrient availability in each of these treatments was investigated at a range of pH values (5,0 to 9,0). In each case, the pH of the culture solution was adjusted with either 1N HCl or 1N NaOH. Cultures were grown in plastic dishes containing 450 ml of nutrient solution and the solutions were replaced daily by siphoning. Each treatment was replicated eight times.

Initially, each dish was inoculated with approximately 1,0 g of *A. filiculoides* plants and growth rates were measured at four-day intervals for a period of 20 days. Nitrogenase activity was measured on the twentieth day using the acetylene reduction assay (Section 5.3.1.1) and all harvested *A. filiculoides* material was dried and chemically analysed (see Section 2.4.1 for details of methods).

#### 6.2.6 Field studies

Chemical analyses of the water samples collected monthly at each of the twelve sampling sites were used to assess the availability of nutrients to *A. filiculoides* growing in the study area. Sample collection techniques and the analytical methods employed have been described in Section 2.4.2.

Water samples collected beneath an established mat of *A. filiculoides* had significantly higher nutrient concentrations than samples from open-water stations. The  $\overline{\text{RGR}}$  of *A. filiculoides* in these two situations was therefore compared, using the floating frames described in Section 2.5.1. At station 9, two frames, each containing 20 perforated dishes were firmly fixed in an open water area and a further two frames were fixed within an established mat. A low (50 cm high) fence of 5 mm aperture gauze wire was strung around each set of apparatus to prevent the movement of *A. filiculoides* plants towards or away from the frames, thereby maintaining either mat or open water conditions.

Each of the 40 dishes in the two treatments was inoculated with a weighed amount (approximately 1,0 g) of *A. filiculoides* plants at the start of the experiment. Every four days the contents of five randomly-chosen dishes were harvested, weighed and chemically analysed. On the twentieth day, nitrogenase activity was measured before harvesting using the acetylene reduction assay (Section 5.3.1.1). Attempts to tag individual plants within an established mat were unsuccessful due to fragmentation of plant material.

In addition to the above, nutrient-enrichment experiments were carried out in the field to determine whether any nutrient or combination of nutrients was limiting the growth of *A. filiculoides*. Water samples from stations 9 and 10 (farm dam and riverine sites, respectively) were spiked with aliquots from stock solutions of individual nutrients and placed in plastic dishes. Each dish contained 450 ml of solution, and was inoculated with a weighed sample of *A. filiculoides* before being placed *in situ* in the frame described in Section 2.5.1. The water in each dish (with or without added nutrients) was replaced every three days to prevent excessive evaporative loss, and all treatments were replicated eight times. Growth rates were measured at seven day intervals for a period of 21 days and compared with the  $\overline{\text{RGR}}$  values of *A. filiculoides* plants grown in stream or dam water lacking supplementary nutrients. Nitrogenase activity was measured on the twenty-first day with the acetylene reduction assay (Section 5.3.1.1).

#### 6.2.7 Measurement of nutrient release from decaying *A. filiculoides* plants

The decomposition of aquatic plants results in rapid loss of mass and nutrients to the water (Boyd, 1970, 1971; Planter, 1970; Jewell, 1971). Inundation of previously-desiccated plants and the decomposition of senescent plants beneath established mats must therefore provide a large source of nutrients for the growth of *A. filiculoides*. The rates of nutrient loss in both of the above situations were therefore investigated by studying the rates of nutrient loss from desiccated and fresh *A. filiculoides* material in the field over a period of 30 days. Since most decomposition appeared to take place in the dark, the experiments were conducted beneath an established *A. filiculoides* mat at station 10. Particular

attention was paid to nutrient loss in the first few hours following inundation, as recommended by Boyd (1970).

Samples of desiccated *A. filiculoides* material were collected from a dried-out pool near station 11 whilst fresh material was collected from a riverine site at station 10. Water was removed by gentle blotting with a towel and subsamples (approximately 50 g of fresh plants or 5 g of desiccated plants) were rapidly weighed and carefully transferred to nylon gauze decomposition bags. Each bag measured 30 x 30 cm with a 1 mm mesh diameter. Four bags each of fresh and desiccated material were fastened to each of 12 metal stakes which were then placed in the water so that all the bags were held in position some 10 cm beneath the *A. filiculoides* mat. One stake and its bags were harvested at 2, 4, 6, 12, 24 and 48 hours and 5, 10, 15, 20, 25 and 30 days after initiation of the experiment. On harvesting, the bags were immediately rinsed with distilled water and stored on ice in numbered plastic bags during transport to the laboratory. In the laboratory, the contents of each bag were dried at 105 °C for 48 hours in a forced-draught oven, weighed, ground and analysed for nutrient content (Section 2.4.1). Four separate samples of desiccated and fresh *A. filiculoides* plants were weighed, dried, reweighed, ground and analysed as above to determine the initial fresh mass/dry mass ratios and nutrient content, before the start of the experiment.

### 6.3 RESULTS

#### 6.3.1 Nutrient availability in the field

The results of the chemical analysis of water samples collected between February 1971 and May 1974 from 12 sites in the study area are summarized in Tables 16 and 17. The sums of all cationic or anionic concentrations at the different sampling sites varied between 1,44 and 4,05 milliequivalents per litre, with the lowest values recorded in summer and highest in winter. Inspection of the results revealed that all cation and anion analyses were within 5 % of chemical balance. At all sampling stations, the ionic concentration of cations or anions in milliequivalents per litre was closely correlated with electrical conductivity ( $r = 0,996$ ;  $n = 120$ ). Stations 1 to 4 on the northern (Orange Free State) bank of the Hendrik Verwoerd Dam had generally lower levels of dissolved cations and anions

TABLE 16 : Variation in concentrations of dissolved, adsorbed and total cations in the surface waters at the twelve sampling stations. Results are given as range of concentrations ( $\text{mg. l}^{-1}$ ) found from February 1971 to May 1974, as well as mean winter ( $\bar{W}$  : May - July ) and mean summer ( $\bar{S}$  : Nov. - Jan. ) concentrations.

	Station 1			Station 2			Station 3			Station 4			Station 5			Station 6			
	range	$\bar{W}$	$\bar{S}$	range	$\bar{W}$	$\bar{S}$	range	$\bar{W}$	$\bar{S}$	range	$\bar{W}$	$\bar{S}$	range	$\bar{W}$	$\bar{S}$	range	$\bar{W}$	$\bar{S}$	
DISSOLVED CATIONS	Na	6,02 - 12,71	11,06	6,70	5,34 - 11,98	10,54	7,12	5,27 - 12,20	11,12	5,95	6,71 - 13,03	12,45	7,30	5,23 - 12,07	11,47	6,02	6,72 - 14,15	12,27	7,70
	K	1,72 - 3,93	3,59	1,88	1,65 - 3,87	3,73	1,87	1,82 - 3,85	3,68	2,02	1,63 - 3,81	3,59	1,99	1,47 - 3,92	3,71	1,88	1,63 - 3,99	3,53	2,04
	Ca	10,17 - 19,25	18,07	11,99	8,25 - 12,36	10,15	9,12	8,07 - 12,48	10,20	9,11	8,04 - 10,12	9,31	8,87	7,10 - 10,24	9,28	7,99	9,84 - 14,93	12,86	10,65
	Mg	8,98 - 17,17	15,41	9,68	8,72 - 17,26	15,93	9,71	8,69 - 17,54	16,11	9,24	8,80 - 18,02	17,13	9,35	7,93 - 17,09	16,01	8,89	9,27 - 18,71	16,92	11,28
	Fe	0,01 - 0,18	0,12	0,03	0,01 - 0,36	0,21	0,06	0,00 - 0,33	0,23	0,06	0,00 - 0,19	0,13	0,04	0,01 - 0,27	0,19	0,05	0,01 - 0,26	0,16	0,02
	Mn	0,02 - 0,29	0,11	0,04	0,02 - 0,38	0,14	0,05	0,01 - 0,38	0,16	0,04	0,00 - 0,26	0,15	0,03	0,02 - 0,33	0,13	0,04	0,02 - 0,23	0,12	0,02
	Cu	0,00 - 0,13	0,11	0,05	0,01 - 0,23	0,12	0,04	0,00 - 0,17	0,11	0,03	0,01 - 0,20	0,11	0,05	0,01 - 0,31	0,13	0,04	0,00 - 0,10	0,07	0,01
Zn	0,01 - 0,16	0,05	0,04	0,02 - 0,21	0,07	0,03	0,01 - 0,19	0,07	0,05	0,02 - 0,22	0,09	0,02	0,02 - 0,28	0,11	0,04	0,00 - 0,08	0,04	0,01	
ADSORBED CATIONS	Na	2,13 - 6,21	2,77	4,19	1,98 - 5,93	2,83	5,11	2,14 - 5,84	2,75	4,92	1,17 - 5,54	2,01	5,07	2,20 - 8,21	2,95	6,93	2,28 - 6,79	2,52	6,38
	K	1,41 - 2,20	1,53	2,08	1,14 - 2,31	1,32	2,01	1,19 - 2,35	1,51	2,10	1,04 - 2,28	1,32	2,09	1,02 - 2,83	1,33	2,45	1,11 - 2,39	1,34	2,22
	Ca	0,78 - 5,11	1,64	4,12	1,01 - 6,98	1,87	4,77	1,03 - 6,29	1,75	4,58	0,72 - 4,29	1,64	3,24	1,03 - 6,81	1,37	4,92	1,34 - 7,12	1,42	6,75
	Mg	3,17 - 5,82	3,81	5,40	3,25 - 5,91	3,52	5,58	3,21 - 6,03	3,98	4,77	3,43 - 5,88	4,10	5,26	2,92 - 5,89	3,56	5,33	3,12 - 6,55	3,40	6,19
	Fe	0,01 - 0,13	0,02	0,10	0,00 - 0,22	0,04	0,13	0,00 - 0,20	0,05	0,16	0,00 - 0,17	0,02	0,08	0,00 - 0,20	0,02	0,09	0,00 - 0,19	0,03	0,08
	Mn	0,01 - 0,18	0,02	0,13	0,00 - 0,23	0,02	0,16	0,01 - 0,27	0,03	0,15	0,01 - 0,15	0,02	0,11	0,00 - 0,17	0,02	0,14	0,01 - 0,14	0,02	0,08
	Cu	0,00 - 0,12	0,02	0,09	0,01 - 0,17	0,02	0,12	0,00 - 0,15	0,02	0,10	0,00 - 0,15	0,01	0,11	0,01 - 0,19	0,02	0,12	0,01 - 0,08	0,02	0,05
Zn	0,00 - 0,15	0,01	0,06	0,00 - 0,19	0,03	0,08	0,00 - 0,16	0,02	0,09	0,01 - 0,13	0,02	0,07	0,01 - 0,15	0,02	0,08	0,00 - 0,03	0,01	0,01	
TOTAL CATIONS	Na	8,15 - 18,92	13,83	10,89	7,32 - 17,91	13,37	12,23	7,41 - 18,04	13,87	10,87	7,88 - 18,57	14,46	12,37	7,43 - 20,28	14,42	12,95	9,00 - 20,94	14,79	14,08
	K	3,13 - 6,13	5,12	3,96	2,79 - 6,18	5,05	3,88	3,01 - 6,20	5,19	4,12	2,67 - 6,09	4,91	4,08	2,49 - 6,75	5,04	4,33	2,74 - 6,38	4,87	4,26
	Ca	7,95 - 14,36	19,71	12,11	9,26 - 19,34	12,02	13,89	9,10 - 18,77	11,95	13,69	8,76 - 14,41	10,95	12,11	8,13 - 17,05	10,65	12,91	11,18 - 22,05	14,28	17,40
	Mg	12,15 - 22,99	19,22	15,08	11,97 - 23,17	19,45	15,29	11,90 - 23,57	20,09	14,01	12,23 - 23,90	21,23	14,61	10,85 - 22,98	19,57	14,22	12,39 - 25,26	20,32	17,47
	Fe	0,02 - 0,31	0,14	0,13	0,01 - 0,58	0,25	0,19	0,00 - 0,53	0,28	0,22	0,00 - 0,36	0,15	0,12	0,01 - 0,47	0,21	0,14	0,01 - 0,45	0,19	0,10
	Mn	0,03 - 0,47	0,13	0,17	0,02 - 0,61	0,16	0,21	0,02 - 0,65	0,19	0,19	0,01 - 0,41	0,17	0,14	0,02 - 0,50	0,15	0,18	0,03 - 0,37	0,14	0,10
	Cu	0,01 - 0,25	0,13	0,14	0,02 - 0,40	0,14	0,16	0,00 - 0,32	0,13	0,13	0,01 - 0,36	0,12	0,16	0,02 - 0,50	0,15	0,16	0,01 - 0,18	0,09	0,06
Zn	0,01 - 0,31	0,06	0,10	0,02 - 0,40	0,10	0,11	0,01 - 0,35	0,09	0,14	0,03 - 0,35	0,11	0,09	0,03 - 0,43	0,13	0,12	0,00 - 0,11	0,05	0,02	

TABLE 16 : (Continued).

	Station 7			Station 8			Station 9			Station 10			Station 11			Station 12			
	range	$\bar{W}$	$\bar{S}$	range	$\bar{W}$	$\bar{S}$	range	$\bar{W}$	$\bar{S}$	range	$\bar{W}$	$\bar{S}$	range	$\bar{W}$	$\bar{S}$	range	$\bar{W}$	$\bar{S}$	
DISSOLVED CATIONS	Na	6,10 - 12,98	12,15	6,92	8,23 - 14,73	13,88	8,97	6,21 - 12,30	11,54	6,99	5,76 - 11,78	11,03	6,72	5,62 - 14,15	13,39	6,87	4,21 - 6,24	5,63	4,75
	K	1,87 - 4,15	3,26	2,19	1,96 - 5,27	4,31	2,72	1,88 - 3,81	3,29	1,96	1,57 - 3,76	3,26	1,97	1,67 - 4,12	3,87	1,95	0,92 - 1,16	1,04	0,99
	Ca	10,27 - 18,87	17,03	12,12	16,83 - 31,27	26,77	21,83	9,82 - 16,71	14,39	10,11	8,13 - 12,37	11,95	8,65	8,65 - 18,75	14,83	9,24	16,36 - 19,24	18,55	16,52
	Mg	9,54 - 21,66	19,27	11,74	15,71 - 26,38	24,28	19,35	9,91 - 22,34	19,11	12,92	9,46 - 20,03	18,52	12,07	9,41 - 21,67	19,11	10,30	18,24 - 23,21	22,64	19,87
	Fe	0,00 - 0,31	0,20	0,03	0,08 - 0,59	0,42	0,13	0,02 - 0,42	0,33	0,05	0,00 - 0,27	0,18	0,03	0,01 - 0,34	0,16	0,04	0,05 - 0,12	0,06	0,08
	Mn	0,00 - 0,36	0,17	0,04	0,09 - 0,53	0,41	0,12	0,00 - 0,36	0,22	0,09	0,01 - 0,20	0,11	0,02	0,01 - 0,31	0,14	0,03	0,00 - 0,02	0,01	0,01
	Cu	0,00 - 0,26	0,14	0,01	0,07 - 0,28	0,21	0,12	0,01 - 0,17	0,10	0,03	0,00 - 0,06	0,04	0,01	0,00 - 0,23	0,07	0,02	0,01 - 0,03	0,01	0,02
Zn	0,00 - 0,10	0,05	0,01	0,05 - 0,16	0,10	0,08	0,00 - 0,14	0,10	0,03	0,01 - 0,07	0,04	0,01	0,01 - 0,14	0,05	0,03	0,00 - 0,03	0,01	0,02	
ADSORBED CATIONS	Na	2,20 - 6,10	2,59	5,47	0,32 - 0,61	0,42	0,37	1,02 - 2,54	1,19	2,31	2,17 - 5,77	2,60	5,33	2,15 - 6,30	2,71	5,36	0,12 - 0,60	0,15	0,27
	K	1,35 - 2,62	1,46	2,53	0,37 - 0,45	0,41	0,39	1,12 - 2,27	1,41	2,09	1,18 - 2,37	1,30	2,07	1,20 - 2,46	1,37	2,05	0,02 - 0,06	0,02	0,03
	Ca	1,47 - 9,93	1,61	9,40	0,14 - 0,19	0,15	0,16	0,98 - 1,24	1,01	1,13	0,97 - 6,42	1,36	5,95	1,38 - 6,10	2,09	4,30	0,40 - 0,82	0,43	0,71
	Mg	3,41 - 7,79	3,52	7,35	0,20 - 0,48	0,23	0,31	1,06 - 2,15	1,10	1,89	2,15 - 6,21	2,68	6,08	2,66 - 7,63	2,93	7,41	0,27 - 0,81	0,38	0,62
	Fe	0,01 - 0,23	0,02	0,10	0,00 - 0,05	0,00	0,03	0,00 - 0,14	0,01	0,06	0,01 - 0,18	0,02	0,09	0,00 - 0,21	0,02	0,08	0,00 - 0,05	0,00	0,02
	Mn	0,01 - 0,22	0,03	0,11	0,00 - 0,02	0,00	0,01	0,00 - 0,11	0,01	0,07	0,01 - 0,15	0,02	0,07	0,01 - 0,22	0,03	0,09	0,00 - 0,01	0,00	0,00
	Cu	0,00 - 0,20	0,01	0,10	0,00 - 0,02	0,00	0,01	0,01 - 0,07	0,01	0,03	0,00 - 0,05	0,01	0,03	0,00 - 0,18	0,01	0,08	0,00 - 0,01	0,01	0,01
Zn	0,01 - 0,09	0,01	0,04	0,00 - 0,01	0,00	0,00	0,00 - 0,04	0,01	0,02	0,00 - 0,05	0,01	0,02	0,00 - 0,12	0,01	0,06	0,00 - 0,01	0,00	0,01	
TOTAL CATIONS	Na	8,30 - 19,08	14,74	12,39	8,55 - 15,34	14,30	9,34	7,23 - 14,84	12,73	9,30	7,93 - 17,55	13,63	12,05	7,77 - 20,45	16,10	12,23	4,33 - 6,84	5,78	5,02
	K	3,22 - 6,77	4,72	4,72	2,33 - 5,72	4,72	3,11	3,00 - 6,08	4,70	4,05	2,75 - 6,13	4,56	4,04	2,87 - 6,58	5,24	4,00	0,94 - 1,22	1,06	1,02
	Ca	11,74 - 28,80	18,64	21,52	16,97 - 31,46	26,92	21,99	10,80 - 17,95	15,40	11,24	9,10 - 18,79	13,31	14,60	10,03 - 24,85	16,92	13,54	16,76 - 20,06	18,98	17,23
	Mg	12,95 - 29,45	22,79	19,09	15,91 - 26,86	24,51	19,66	10,97 - 24,49	20,21	14,81	11,61 - 26,24	21,20	18,15	12,07 - 29,30	22,04	17,71	18,51 - 24,02	23,02	20,49
	Fe	0,01 - 0,54	0,22	0,13	0,08 - 0,64	0,42	0,16	0,02 - 0,56	0,34	0,11	0,01 - 0,45	0,20	0,12	0,01 - 0,55	0,18	0,12	0,05 - 0,17	0,06	0,10
	Mn	0,01 - 0,58	0,20	0,15	0,09 - 0,55	0,41	0,13	0,00 - 0,49	0,23	0,16	0,02 - 0,35	0,13	0,09	0,02 - 0,53	0,17	0,12	0,00 - 0,03	0,01	0,01
	Cu	0,00 - 0,46	0,15	0,11	0,07 - 0,30	0,21	0,13	0,02 - 0,24	0,11	0,06	0,00 - 0,11	0,05	0,04	0,00 - 0,41	0,08	0,10	0,01 - 0,04	0,02	0,03
Zn	0,01 - 0,19	0,06	0,05	0,05 - 0,17	0,10	0,08	0,00 - 0,18	0,11	0,05	0,01 - 0,12	0,05	0,03	0,01 - 0,26	0,06	0,09	0,00 - 0,04	0,01	0,03	

TABLE 17 : Variation in anion and nutrient concentrations in surface waters at the twelve sampling stations. Results are given as range of concentrations ( in  $\text{mg} \cdot \ell^{-1}$  unless otherwise indicated) found from February 1971 to May 1974, as well as mean winter (  $\bar{W}$  : May - July ) and mean summer (  $\bar{S}$  : Nov. - Jan. ) concentrations.

Parameter	Station 1			Station 2			Station 3		
	range	$\bar{W}$	$\bar{S}$	range	$\bar{W}$	$\bar{S}$	range	$\bar{W}$	$\bar{S}$
Suspended solids	41,8 - 253,5	47,5	226,0	32,7 - 268,5	49,2	184,5	26,3 - 252,1	42,0	178,7
pH	7,99 - 8,63	8,05	8,42	7,65 - 8,78	8,01	8,60	7,65 - 8,85	7,95	8,55
Conductivity ( $\mu\text{Mhos} \cdot \text{cm}^{-1}$ )	142 - 283	255	160	130 - 241	215	150	127 - 252	219	141
Total alkalinity ( $\text{mg CaCO}_3 \cdot \ell^{-1}$ )	34,27 - 76,11	68,25	36,80	32,96 - 68,75	58,50	36,75	29,26 - 66,37	59,65	31,37
Sulphate	21,99 - 33,09	30,04	24,74	19,83 - 30,14	27,36	21,89	19,45 - 31,40	28,41	22,54
Chloride	16,27 - 29,12	25,11	18,23	14,77 - 25,25	22,85	16,48	15,10 - 26,05	21,92	17,43
Orthophosphate ( $\text{PO}_4$ )	0,01 - 0,07	0,04	0,02	0,01 - 0,10	0,06	0,03	0,01 - 0,12	0,07	0,03
Total phosphorus	0,02 - 0,11	0,09	0,05	0,03 - 0,17	0,10	0,07	0,03 - 0,20	0,13	0,08
$\text{NH}_4\text{-N}$	0,13 - 1,01	0,72	0,26	0,16 - 0,88	0,65	0,29	0,15 - 0,83	0,62	0,24
$\text{NO}_2\text{-N} + \text{NO}_3\text{-N}$	0,85 - 1,62	1,38	1,04	0,79 - 1,82	1,49	1,07	0,78 - 2,01	1,45	0,99
Soluble organic nitrogen	0,08 - 0,42	0,25	0,14	0,11 - 0,60	0,41	0,22	0,12 - 0,87	0,48	0,27
Total nitrogen	1,06 - 3,05	2,35	1,44	1,06 - 3,30	2,55	1,58	1,05 - 3,71	2,55	1,50

TABLE 17 : (Continued).

Parameter	Station 4			Station 5			Station 6		
	range	$\bar{W}$	$\bar{S}$	range	$\bar{W}$	$\bar{S}$	range	$\bar{W}$	$\bar{S}$
Suspended solids	18,9 - 228,4	29,3	186,5	26,3 - 292,5	30,1	247,4	42,8 - 255,6	49,8	213,5
pH	7,51 - 8,52	7,72	8,35	7,40 - 8,52	7,64	8,26	7,70 - 8,41	7,88	8,24
Conductivity ( $\mu\text{Mhos.cm}^{-1}$ )	128 - 246	227	147	114 - 244	218	129	139 - 282	246	162
Total alkalinity ( $\text{mg CaCO}_3 \cdot \ell^{-1}$ )	30,14 - 69,03	61,27	33,69	32,45 - 68,13	59,92	36,12	35,14 - 77,25	67,44	39,27
Sulphate	15,25 - 33,10	29,16	16,33	11,28 - 31,78	26,45	13,75	14,80 - 37,63	31,68	18,29
Chloride	12,84 - 26,99	23,92	14,27	13,63 - 26,41	22,74	18,46	19,89 - 30,14	27,08	22,05
Orthophosphate ( $\text{PO}_4$ )	0,01 - 0,07	0,05	0,02	0,01 - 0,09	0,04	0,02	0,01 - 0,14	0,08	0,03
Total phosphorus	0,03 - 0,12	0,08	0,04	0,02 - 0,15	0,08	0,03	0,03 - 0,29	0,14	0,07
$\text{NH}_4\text{-N}$	0,10 - 0,26	0,19	0,15	0,09 - 0,24	0,18	0,15	0,12 - 0,63	0,28	0,15
$\text{NO}_2\text{-N} + \text{NO}_3\text{-N}$	0,77 - 1,48	1,15	0,86	0,62 - 1,53	1,24	0,69	0,49 - 1,89	1,44	0,60
Soluble organic nitrogen	0,09 - 0,35	0,24	0,11	0,08 - 0,46	0,32	0,11	0,14 - 0,82	0,37	0,19
Total nitrogen	0,96 - 2,09	1,58	1,12	0,79 - 2,23	1,74	0,95	0,75 - 3,34	2,09	0,94

TABLE 17 : (Continued).

Parameter	Station 7			Station 8			Station 9		
	range	$\bar{W}$	$\bar{S}$	range	$\bar{W}$	$\bar{S}$	range	$\bar{W}$	$\bar{S}$
Suspended solids	49,2 - 349,9	55,7	301,4	48,9 - 85,2	51,4	76,5	35,9 - 204,5	46,3	156,2
pH	7,68 - 8,20	7,85	8,03	8,65 - 9,41	8,76	9,24	7,58 - 9,38	8,09	9,29
Conductivity ( $\mu\text{Mhos.cm}^{-1}$ )	154 - 289	274	178	264 - 383	364	289	159 - 277	256	173
Total alkalinity ( $\text{mg CaCO}_3 \cdot \ell^{-1}$ )	46,91 - 88,07	75,03	53,94	95,25 - 140,18	123,79	97,44	46,30 - 87,85	76,51	51,00
Sulphate	12,16 - 40,12	33,76	18,63	10,82 - 14,67	13,41	11,2	14,80 - 35,91	31,15	18,44
Chloride	14,80 - 36,57	32,50	17,71	32,74 - 46,09	44,80	34,53	13,22 - 30,08	24,46	17,39
Orthophosphate ( $\text{PO}_4$ )	0,01 - 0,07	0,03	0,01	0,12 - 2,36	0,27	2,18	0,10 - 2,65	0,42	2,31
Total phosphorus	0,02 - 0,12	0,07	0,03	0,19 - 3,83	0,70	3,29	0,15 - 2,95	0,68	2,54
$\text{NH}_4\text{-N}$	0,08 - 0,26	0,19	0,11	0,27 - 0,94	0,71	0,44	0,27 - 0,98	0,39	0,77
$\text{NO}_2\text{-N} + \text{NO}_3\text{-N}$	0,83 - 2,03	1,69	0,97	1,68 - 7,98	3,77	6,80	0,42 - 6,35	0,60	5,10
Soluble organic nitrogen	0,07 - 0,32	0,21	0,09	0,29 - 1,85	0,62	1,41	0,26 - 1,14	0,47	0,98
Total nitrogen	0,98 - 2,61	2,09	1,17	2,24 - 10,77	5,10	8,65	0,95 - 8,47	1,46	6,85

TABLE 17 : (Continued).

Parameter	Station 10			Station 11			Station 12		
	range	$\bar{W}$	$\bar{S}$	range	$\bar{W}$	$\bar{S}$	range	$\bar{W}$	$\bar{S}$
Suspended solids	43,2 - 299,8	50,6	267,4	51,2 - 374,8	62,1	295,6	11,6 - 39,5	15,5	24,8
pH	8,86 - 9,31	8,89	9,24	7,95 - 8,82	8,10	8,40	7,90 - 8,04	7,95	8,02
Conductivity ( $\mu\text{Mhos.cm}^{-1}$ )	128 - 276	244	158	136 - 302	278	151	232 - 299	285	243
Total alkalinity ( $\text{mg CaCO}_3 \cdot \ell^{-1}$ )	53,19 - 87,89	82,71	55,62	52,83 - 104,17	98,15	54,90	82,16 - 112,45	108,52	89,40
Sulphate	10,65 - 20,25	18,61	12,31	9,49 - 26,11	22,62	11,43	18,54 - 26,14	24,21	19,89
Chloride	12,14 - 26,50	20,10	13,97	10,80 - 24,62	20,56	12,86	11,71 - 15,32	14,26	12,66
Orthophosphate ( $\text{PO}_4$ )	0,01 - 0,72	0,05	0,65	0,02 - 0,10	0,05	0,03	0,01 - 0,04	0,03	0,02
Total phosphorus	0,04 - 1,01	0,19	0,77	0,05 - 0,36	0,21	0,07	0,01 - 0,09	0,05	0,03
$\text{NH}_4\text{-N}$	0,14 - 0,46	0,17	0,33	0,09 - 0,41	0,26	0,12	0,02 - 0,18	0,12	0,08
$\text{NO}_2\text{-N} + \text{NO}_3\text{-N}$	0,80 - 2,15	1,58	0,97	0,21 - 1,68	1,25	0,30	0,20 - 0,47	0,41	0,26
Soluble organic nitrogen	0,15 - 0,35	0,20	0,30	0,10 - 0,38	0,19	0,13	0,09 - 0,19	0,14	0,11
Total nitrogen	1,09 - 2,96	1,95	1,60	0,40 - 2,47	1,70	0,55	0,31 - 0,84	0,67	0,45

than stations 5 to 12, located on the southern (Cape Province) bank of the impoundment.

Throughout the study, the dominance order of dissolved major cations at stations 1 to 3 and 6 to 12 was  $Mg > Ca > Na > K$ . Stations 4 and 5 displayed a similar cation dominance order during the summer months, but this changed to  $Mg > Na > Ca > K$  during winter. The ratio of monovalent to divalent cations ( $Na + K / Ca + Mg$ ; Talling and Talling, 1965) showed little variation about a mean of 0,30 for stations 1 to 11, but was quite different (0,10) at station 12, the eye of a spring in a dolerite ridge. The anion dominance order was  $HCO_3 > Cl > SO_4$  at stations 1 to 11 and  $HCO_3 > SO_4 > Cl$  at station 12 throughout the study.

Concentrations of suspended solids varied from  $11,6 \text{ mg} \cdot \text{l}^{-1}$  in winter to  $374,8 \text{ mg} \cdot \text{l}^{-1}$  in summer at the 12 sampling stations. The lowest values were recorded at station 12 located at a spring in a dolerite ridge. Winter values were always lower than summer values at every sampling site. In winter the suspended material was predominantly detritus while silt formed the major component of the suspended material in summer.

Adsorbed major cations, measured by the ammonium acetate extraction method, accounted for a large proportion of the total cations available for plant growth. The proportion of total cations formed by the adsorbed fraction varied from 17 % in winter to 39 % in summer. Thus, despite a decrease in dissolved cation concentrations during high summer flows, the higher summer concentration of adsorbed cations ensured that the concentration of total cations at any one station showed only small seasonal variation (Table 16). With regard to the individual cations, the proportion of the total concentration of each cation adsorbed onto particulate material in winter was usually less than half the corresponding proportion in summer (Table 18). The greatest degree of adsorption was shown by K ions, 27,9 % and 52,2 % of the total K concentration being adsorbed to particulates during winter and summer, respectively. The least adsorption was shown by Ca ions, with 12,0 % and 35,8 % adsorbed during winter and summer, respectively.

TABLE 18 - Seasonal proportions of each major cation adsorbed onto particulate material, at all riverine stations, i.e. stations 1 to 7, 10 and 11. (The values were calculated as percentages and are given as the mean of the riverine stations  $\pm$  two standard errors of the mean.)

Season	Cation			
	Mg	Ca	Na	K
Winter	17,0 $\pm$ 1,80	12,0 $\pm$ 1,94	18,4 $\pm$ 1,46	27,9 $\pm$ 1,26
Summer	36,3 $\pm$ 1,74	35,8 $\pm$ 3,46	44,0 $\pm$ 2,74	52,2 $\pm$ 1,14

Anion concentrations in the riverine stations showed slight seasonal variation, though very little variation (usually < 5 %) between stations within the same season. In winter, the concentrations of all anions were greater than the summer concentrations. Despite this seasonal variation in concentration, the ratio of chloride to sulphate (by milliequivalents) at all sampling stations remained almost constant at 1,05.

Total alkalinity, representing the anions  $\text{HCO}_3^-$  and  $\text{CO}_3^{=}$ , accounted for up to 64 % of the total anion concentration at all sampling stations. As was expected, the pH of the water at all sampling stations was alkaline and varied from 7,40 to 9,41. The highest values were recorded at stations 8 and 9, two artificial reservoirs. Water pH values in winter were always lower than the summer values at all sampling stations.

The minor cations Fe, Mn, Cu and Zn were present at all sampling stations but only in very low concentrations, occasionally below the detection limit of the analytical methods used. The concentration of these cations was lowest in summer during high river flows. Significant proportions of these cations (15 to 65 %) were adsorbed onto particulate material, particularly silt, in summer (Table 16).

The concentrations of  $\text{PO}_4\text{-P}$  and total phosphorus at the riverine sampling stations showed very little variation during this study but were higher in winter and lower during the summer months (Table 17). Low  $\text{PO}_4\text{-P}$  concentrations (0,01 to 0,04  $\text{mg}\cdot\text{l}^{-1}$ ) were always found when water flow was

highest and higher concentrations ( $0,05$  to  $0,12 \text{ mg.l}^{-1}$ ) were found during periods of low flow. Concentrations of the two phosphorus fractions were considerably higher (up to  $2,31$  and  $3,29 \text{ mg.l}^{-1}$ , respectively) at stations 8 and 9, the two reservoir stations, particularly during summer.

At the riverine sampling stations, concentrations of inorganic nitrogen  $\text{NH}_4\text{-N}$  and  $\text{NO}_3\text{-N}$  plus  $\text{NO}_2\text{-N}$ ) were very similar and tended to be higher in winter. At the two reservoir stations, the concentrations of these two nitrogen forms was much greater, displaying summer maxima and winter minima. Station 12, located at the spring, had consistently low levels of both phosphorus and nitrogen.

### 6.3.2 Culture media - Selection of an optimum medium and comparisons with field conditions

As mentioned in Section 6.2.1, the first eight media ('A' to 'H') were tested at the beginning of this study to find the optimum medium for routine studies of growth and nitrogenase activity by *A. filiculoides*. The last two media, 'I' and 'J', were tested at a later stage of the study for comparative purposes only.

In the three concentration levels of the ten nutrient media tested, the concentration of total dissolved salts (TDS) varied from  $41$  to  $2\ 220 \text{ mg.l}^{-1}$ , a range of concentrations which was far greater than the range of  $152$  to  $279 \text{ mg.l}^{-1}$  encountered in the study area. The values of  $\overline{\text{RGR}}$  and nitrogenase activity by *A. filiculoides* in the different media are shown in Tables 19 and 20.

In media 'A' to 'H', *A. filiculoides*  $\overline{\text{RGR}}$  values varied from  $0,113$  to  $0,298 \text{ g.g}^{-1}.\text{day}^{-1}$ . The lowest  $\overline{\text{RGR}}$  values ( $0,113$  to  $0,128 \text{ g.g}^{-1}.\text{day}^{-1}$ ) were recorded in those media having the highest TDS concentration ('A' and 'D'). The highest  $\overline{\text{RGR}}$  values ( $0,268$  to  $0,298 \text{ g.g}^{-1}.\text{day}^{-1}$ ) were recorded at TDS concentrations ranging from  $178$  to  $407 \text{ mg.l}^{-1}$  (media 'C', 'E', 'F', 'G' and 'H'). Slightly higher TDS concentrations ( $814 \text{ mg.l}^{-1}$ : medium 'E' at double-strength) depressed  $\overline{\text{RGR}}$  values by up to 10 %. Consistently higher  $\overline{\text{RGR}}$  values were obtained with medium 'F' at both full- and double-strength. Examination of the  $\overline{\text{RGR}}$  values obtained with media 'I' and 'J' showed that both media compared favourably with medium 'F', though the

TABLE 19 - Mean relative growth rates (RGR:  $\overline{\text{g.g.}}^{-1} \cdot \text{day}^{-1}$ ) of *Azolla filiculoides* grown in ten different nutrient media<sup>a</sup>, at three concentration levels and three different micro-nutrient formulations. (Results are given as mean of ten replicates  $\pm$  two standard errors of the mean.)

Macronutrient Concentration	Micro-nutrient Soln.**	Nutrient Medium				
		A	B	C	D	E
(½ X)	A	0,176 $\pm$ 0,026	0,149 $\pm$ 0,015	0,254 $\pm$ 0,027	0,169 $\pm$ 0,044	0,250 $\pm$ 0,019
	B	0,171 $\pm$ 0,033	0,158 $\pm$ 0,020	0,268 $\pm$ 0,032	0,182 $\pm$ 0,028	0,248 $\pm$ 0,032
	C	0,179 $\pm$ 0,020	0,143 $\pm$ 0,016	0,249 $\pm$ 0,019	0,177 $\pm$ 0,026	0,257 $\pm$ 0,026
(1 X)	A	0,142 $\pm$ 0,027	0,177 $\pm$ 0,026	0,166 $\pm$ 0,016	0,158 $\pm$ 0,026	0,259 $\pm$ 0,037
	B	0,153 $\pm$ 0,031	0,181 $\pm$ 0,026	0,158 $\pm$ 0,021	0,162 $\pm$ 0,040	0,268 $\pm$ 0,029
	C	0,149 $\pm$ 0,019	0,184 $\pm$ 0,014	0,160 $\pm$ 0,020	0,149 $\pm$ 0,039	0,253 $\pm$ 0,042
(2 X)	A	0,122 $\pm$ 0,020	0,219 $\pm$ 0,030	0,150 $\pm$ 0,021	0,128 $\pm$ 0,022	0,207 $\pm$ 0,042
	B	0,119 $\pm$ 0,014	0,207 $\pm$ 0,018	0,150 $\pm$ 0,030	0,113 $\pm$ 0,014	0,202 $\pm$ 0,029
	C	0,124 $\pm$ 0,015	0,209 $\pm$ 0,025	0,143 $\pm$ 0,026	0,119 $\pm$ 0,032	0,205 $\pm$ 0,036

\* Specifications of each nutrient medium are given in Table 13

\*\* Micronutrient solutions listed in Table 14

TABLE 19 (continued)

Macronutrient Concentration	Micro-nutrient Soln.	Nutrient Medium				
		F	G	H	I	J
(½ X)	A	0,227 ± 0,032	0,187 ± 0,021	0,174 ± 0,018	0,224 ± 0,029	0,269 ± 0,040
	B	0,240 ± 0,046	0,196 ± 0,026	0,170 ± 0,027	0,237 ± 0,031	0,275 ± 0,023
	C	0,235 ± 0,025	0,181 ± 0,019	0,180 ± 0,019	0,230 ± 0,020	0,271 ± 0,038
(1 X)	A	0,288 ± 0,029	0,251 ± 0,035	0,210 ± 0,030	0,265 ± 0,032	0,301 ± 0,042
	B	0,298 ± 0,021	0,260 ± 0,040	0,207 ± 0,014	0,272 ± 0,019	0,310 ± 0,036
	C	0,284 ± 0,038	0,242 ± 0,028	0,193 ± 0,029	0,261 ± 0,026	0,301 ± 0,029
(2X)	A	0,288 ± 0,041	0,279 ± 0,026	0,228 ± 0,018	0,278 ± 0,029	0,232 ± 0,036
	B	0,295 ± 0,026	0,275 ± 0,037	0,214 ± 0,041	0,282 ± 0,038	0,248 ± 0,039
	C	0,280 ± 0,035	0,282 ± 0,018	0,237 ± 0,035	0,269 ± 0,032	0,240 ± 0,025

TABLE 20 - Nitrogenase activity (nmoles  $C_2H_4$  (g. fresh wt)<sup>-1</sup>.hr<sup>-1</sup>) of *Azolla filiculoides* grown in ten different nutrient media\*, at three concentration levels and three different micronutrient formulations. (Results are given as mean of four replicates  $\pm$  two standard errors of the mean.)

Macronutrient Concentration	Micro nutrient Soln.**	Nutrient Medium									
		A	B	C	D	E	F	G	H	I	J
(½ X )	A	1 240 $\pm$ 74	782 $\pm$ 42	1 880 $\pm$ 92	1 477 $\pm$ 88	2 611 $\pm$ 127	2 305 $\pm$ 79	1 700 $\pm$ 103	1 488 $\pm$ 46	2 185 $\pm$ 92	2 700 $\pm$ 109
	B	1 198 $\pm$ 92	795 $\pm$ 35	1 927 $\pm$ 110	1 546 $\pm$ 104	2 620 $\pm$ 142	2 372 $\pm$ 88	1 758 $\pm$ 87	1 520 $\pm$ 87	2 242 $\pm$ 103	2 690 $\pm$ 89
	C	1 216 $\pm$ 85	770 $\pm$ 47	1 903 $\pm$ 89	1 503 $\pm$ 93	2 587 $\pm$ 102	2 280 $\pm$ 101	1 719 $\pm$ 88	1 459 $\pm$ 103	2 209 $\pm$ 66	2 656 $\pm$ 120
(1 X)	A	876 $\pm$ 56	1 580 $\pm$ 58	982 $\pm$ 48	1 230 $\pm$ 101	2 603 $\pm$ 114	2 704 $\pm$ 122	2 477 $\pm$ 117	1 927 $\pm$ 62	2 530 $\pm$ 76	2 896 $\pm$ 141
	B	913 $\pm$ 49	1 565 $\pm$ 87	1 015 $\pm$ 63	1 214 $\pm$ 76	2 650 $\pm$ 78	2 780 $\pm$ 120	2 525 $\pm$ 146	1 946 $\pm$ 83	2 549 $\pm$ 104	2 925 $\pm$ 157
	C	868 $\pm$ 83	1 579 $\pm$ 77	1 004 $\pm$ 55	1 186 $\pm$ 82	2 587 $\pm$ 121	2 695 $\pm$ 82	2 502 $\pm$ 88	1 875 $\pm$ 77	2 515 $\pm$ 82	2 902 $\pm$ 122
(2 X)	A	467 $\pm$ 76	1 792 $\pm$ 96	890 $\pm$ 38	548 $\pm$ 37	2 286 $\pm$ 65	2 690 $\pm$ 106	2 620 $\pm$ 137	2 005 $\pm$ 142	2 605 $\pm$ 107	2 170 $\pm$ 113
	B	492 $\pm$ 60	1 806 $\pm$ 112	912 $\pm$ 76	493 $\pm$ 52	2 319 $\pm$ 86	2 750 $\pm$ 141	2 598 $\pm$ 95	1 988 $\pm$ 87	2 641 $\pm$ 92	2 289 $\pm$ 90
	C	480 $\pm$ 52	1 778 $\pm$ 87	910 $\pm$ 71	521 $\pm$ 38	2 301 $\pm$ 58	2 714 $\pm$ 136	2 650 $\pm$ 118	2 015 $\pm$ 109	2 575 $\pm$ 69	2 256 $\pm$ 140

\* Specifications of each nutrient medium are given in Table 13

\*\* Micronutrient solutions listed in Table 14

full-strength version of medium 'J' gave rise to marginally higher  $\overline{\text{RGR}}$  values (up to  $0,310 \text{ g.g}^{-1}.\text{day}^{-1}$ : Table 19). At any one concentration level in a medium, *A. filiculoides*  $\overline{\text{RGR}}$  values in the treatments receiving different micronutrient formulations were not significantly different ( $P > 0,05$ ). However, *A. filiculoides*  $\overline{\text{RGR}}$  values in those treatments receiving the micronutrient combination 'B' were slightly higher in 19 of the 30 trials (63 %).

Nitrogenase activity in the different nutrient media produced trends very similar ( $r^2 = 0,902$ ;  $n = 60$ ) to those obtained for  $\overline{\text{RGR}}$  (Table 20). Here, nitrogenase activity varied from 467 to 2 780 nmoles  $\text{C}_2\text{H}_4$  produced per gramme fresh weight per hour in media 'A' to 'H'. High and low concentrations of TDS also depressed nitrogenase activity. Media 'I' and 'J' again compared favourably with medium 'F', and as before, the full-strength version of medium 'J' gave rise to marginally higher nitrogenase activity values (Table 20).

On the basis of the results presented in Tables 19 and 20, the full-strength version of the macronutrient medium 'F' and the micronutrient combination 'B' were selected for further optimization and routine laboratory use.

### 6.3.3 Optimum levels of macro- and micronutrients

The range of concentrations of each macronutrient, obtained by serial dilutions of individual nutrient stock solutions (Table 21), were far greater than those found in the field. During the acclimatization period of 21 days, the lower nutrient concentrations caused the development of deficiency symptoms whilst the highest concentrations inhibited growth.

TABLE 21 - Concentration of major elements in different dilutions of macronutrient medium 'F' (mg.l<sup>-1</sup>)

Element	Concentration of element							
	1/32 X	1/16 X	1/8 X	1/4 X	1/2 X	1 X	2 X	4 X
Ca	0,25	0,5	1	2	4	8	16	32
Mg	0,312	0,625	1,25	2,5	5	10	20	40
Na	1,594	3,188	6,375	12,75	25,5	51	102	204
K	0,25	0,5	1	2	4	8	16	32
S	0,406	0,812	1,625	3,25	6,5	13	26	52
P	0,203	0,406	0,812	1,625	3,25	6,5	13	26
Cl	2,625	5,25	10,5	21	42	84	168	336
Fe	0,125	0,25	0,5	1	2	4	8	16

The  $\overline{\text{RGR}}$  values and nitrogenase activity of *A. filiculoides* grown at the different levels of individual nutrients are shown in Tables 22 and 23 respectively.

TABLE 22 - Growth rates of *A. filiculoides* grown with varying concentrations of individual macronutrients. (Results are expressed as percentages of the  $\overline{\text{RGR}}$  in full-strength (1 X) macronutrient medium 'F', which served as a control.)

Element	Concentration of element							
	1/32 X	1/16 X	1/8 X	1/4 X	1/2 X	1 X	2 X	4 X
Ca	3	5	7	36	68	100	106	76
Mg	21	31	59	82	95	100	102	90
Na	82	88	93	97	99	100	99	90
K	12	24	36	57	81	100	100	99
S	68	76	84	94	101	100	101	100
P	68	91	99	100	103	100	100	89
Cl	80	88	94	100	99	100	102	95
Fe	70	92	102	103	103	100	87	65

TABLE 23 - Nitrogenase activity of *A. filiculoides* grown with varying concentrations of individual macronutrients. (Results are expressed as percentages of nitrogenase activity in full-strength (1 X) macronutrient medium 'F', which served as a control.)

Element	Concentration of element							
	1/32 X	1/16 X	1/8 X	1/4 X	1/2 X	1 X	2 X	4 X
Ca	1	2	4	18	81	100	110	63
Mg	16	28	50	69	85	100	109	85
Na	78	86	94	97	99	100	101	92
K	4	7	11	36	89	100	109	88
S	69	82	93	99	102	100	106	101
P	61	86	100	103	105	100	101	91
Cl	82	90	97	101	100	100	103	99
Fe	64	87	101	108	106	100	82	56

At the lower concentrations of Ca, K and Mg, the  $\overline{\text{RGR}}$  of *A. filiculoides* was extremely low (< 30 % of the  $\overline{\text{RGR}}$  in full-strength medium: Table 22). Increased concentrations of these elements resulted in an increase in  $\overline{\text{RGR}}$ , with the optimum concentration located between 1 X and 2 X the normal medium concentration. Higher (4 X) levels of these elements caused a decrease in  $\overline{\text{RGR}}$  which was particularly evident in the case of Ca. Decreasing the concentrations of Na,  $\text{SO}_4$  and Cl down to 1/32 X resulted in a decrease in  $\overline{\text{RGR}}$  down to 68 % ( $\text{SO}_4$ ), 80 % (Cl) and 82 % (Na) of the  $\overline{\text{RGR}}$  in the complete medium. However, each of these three nutrients was capable of supporting optimum growth of *A. filiculoides* over a wide concentration range. *Azolla filiculoides* was also found to be capable of rapid growth in a wide range of P (1/8 X to 2 X) and Fe (1/8 X to 1 X) concentrations, but both high and low levels of these two nutrients caused a decrease in the  $\overline{\text{RGR}}$  of *A. filiculoides*.

The trends in nitrogenase activity (Table 23) were almost identical to those described above for *A. filiculoides*  $\overline{\text{RGR}}$  values. The exception was K, which was almost as effective as Ca in reducing nitrogenase activity at low concentrations.

After 20 days, nutrient-deficiency symptoms were clearly evident in P-, Ca-, Mg-, K- and Fe-deficient plants at the lowest concentrations used. With the exception of those plants grown in the highest Ca and Fe concentrations, all the other *A. filiculoides* plants remained green and healthy, and developed large fronds (25 to 35 mm diameter). At the highest Ca and Fe concentrations (4 X), *A. filiculoides* plants became chlorotic with small necrotic spots on a few of the dorsal leaf lobes. At the lowest concentrations of Ca, Mg, Fe and K (1/32 X), all the fronds turned pale yellowish-green and developed large necrotic spots on the dorsal leaf lobes. Furthermore, the fronds were small (5 to 10 mm diameter), compact and were easily disintegrated. *Azolla filiculoides* plants grown at the lowest P concentration (1/32 X) turned pale brownish in colour. The roots of these plants became extraordinarily elongated (up to a 3,5-fold increase in length) and very curled and twisted.

The range of micronutrient concentrations obtained by dilution of individual micronutrient stock solutions (Table 24) generally fell within the range found in the field. Although B and Mo were not routinely analysed in the water samples, occasional analyses showed them to be present at levels of 0,8 to 4,5  $\mu\text{g}.\ell^{-1}$  and 0,5 to 2,2  $\mu\text{g}.\ell^{-1}$  respectively. The  $\overline{\text{RGR}}$  and nitrogenase activity of *A. filiculoides* plants grown at the different levels of individual micronutrients are shown in Tables 25 and 26 respectively. During the acclimatization period of 21 days, the lower concentrations of micronutrients caused the development of slight nutrient-deficiency symptoms. However, foliar symptoms of nutrient deficiency were only evident in the lowest concentrations of B, Co and Mo. These symptoms, identical for the three treatments, consisted of a slight yellowing of the fronds, with the fronds appearing smaller and more compact.

At the lowest concentrations of Co, Cu, Mn, Mo and Zn used, *A. filiculoides*  $\overline{\text{RGR}}$  values were reduced by 12 to 28 % below the control medium (Table 25). The lowest concentration of B caused a 45 % drop in  $\overline{\text{RGR}}$ . When the concentrations of these six elements were increased above 1/32 X, the  $\overline{\text{RGR}}$  values increased, with the optimum concentration of each element (in terms of highest  $\overline{\text{RGR}}$  values) located at approximately half the control medium (1 X) concentration. The highest (4 X) levels of B, Co, Cu and Mo resulted in decreased  $\overline{\text{RGR}}$  values, with the greatest decrease (13 %) occurring for Cu.

TABLE 24 - Concentrations of minor elements in different dilutions of micronutrient combination 'B' ( $\mu\text{g}.\text{l}^{-1}$ )

Element	Concentration of element							
	1/32 X	1/16 X	1/8 X	1/4 X	1/2 X	1 X	2 X	4 X
B	2	4	8	16	32	64	128	256
Mn	1,95	3,89	7,78	15,55	31,1	62,2	124	248
Zn	0,37	0,74	1,47	2,93	5,85	11,7	23,4	46,8
Mo	0,15	0,30	0,60	1,2	2,4	4,8	9,6	19,2
Cu	0,16	0,32	0,64	1,28	2,55	5,1	10,2	20,4
Co	0,15	0,30	0,59	1,18	2,35	4,7	9,4	18,8

TABLE 25 - Growth rates of *A. filiculoides* grown with varying concentrations of individual micronutrients. (Results are expressed as percentages of the RGR in full-strength (1 X) micronutrient combination 'B', which served as control.)

Element	Concentration of element							
	1/32 X	1/16 X	1/8 X	1/4 X	1/2 X	1 X	2 X	4 X
B	55	69	86	99	102	100	100	98
Mn	72	86	95	102	101	100	101	100
Zn	86	92	96	101	102	100	103	100
Mo	78	92	101	106	102	100	99	96
Cu	88	95	99	100	105	100	95	87
Co	78	88	96	102	106	100	100	96

Table 26 - Nitrogenase activity of *A. filiculoides* grown with varying concentrations of individual micronutrients. (Results are expressed as percentages of the nitrogenase activity in full-strength (1 X) micronutrient combination 'B', which served as control.)

Element	Concentration of element							
	1/32 X	1/16 X	1/8 X	1/4 X	1/2 X	1 X	2 X	4 X
B	72	88	97	100	104	100	103	95
Mn	72	89	100	104	101	100	102	102
Zn	93	97	99	100	105	100	102	100
Mo	47	76	99	103	102	100	96	95
Cu	89	97	98	102	100	100	95	85
Co	53	80	91	103	101	100	102	94

The trends in *A. filiculoides* nitrogenase activity (Table 26) were almost identical to those described above for  $\overline{\text{RGR}}$ . The exceptions were a smaller reduction in nitrogenase activity at low B levels and a greater reduction in nitrogenase activity at low Co and Mo levels. Optimum micronutrient levels for nitrogenase activity were also located at approximately half the control medium (1 X) concentration.

Combination of the results obtained on the effects of individual macro- and micronutrient concentrations enabled a modified culture solution to be designed such that each nutrient was supplied at a concentration which supported the highest  $\overline{\text{RGR}}$  and nitrogenase activity values. The details of this modified culture solution (named AZOLLA-I) are given in Table 27. Growth trials with this medium gave very high  $\overline{\text{RGR}}$  values (average  $0,330 \text{ g.g}^{-1} \cdot \text{day}^{-1}$ ) and nitrogenase activity (average  $3\ 215 \text{ nmoles C}_2\text{H}_4$  produced per gramme fresh weight per hour). These rates were approximately 10 % higher than those achieved with the original medium 'F' plus micronutrient combination 'B'.

#### 6.3.4 Hydrological influences and minimum nutrient requirements

In continuous culture, both nutrient concentration and flow rate affected the  $\overline{\text{RGR}}$  values of *A. filiculoides* (Table 28). With the full-strength (1 X) version of Azolla-I medium, high  $\overline{\text{RGR}}$  values were maintained over the whole range of flow rates. At each of the flow rates used,  $\overline{\text{RGR}}$  declined

TABLE 27 - Composition of modified culture solution AZOLLA-I

A. Concentration of macronutrient salts in medium

Salt	mmole.l <sup>-1</sup>
CaCl <sub>2</sub> .2H <sub>2</sub> O	0,4
MgSO <sub>4</sub> .7H <sub>2</sub> O	0,8
K <sub>2</sub> HPO <sub>4</sub>	0,2
Na <sub>2</sub> HPO <sub>4</sub>	0,2
NaCl	2,0
Fe-Na-EDTA	0,02

B. Concentration of elements in medium

Element	mmole.l <sup>-1</sup>	mg.l <sup>-1</sup>
Ca	0,4	16,0
Mg	0,8	19,5
Na	2,4	55,2
K	0,4	15,6
S	0,8	25,6
P	0,4	12,4
Cl	2,8	99,3
Fe	0,02	1,0

C. Concentration of micronutrient salts in medium

Salt	μmole.l <sup>-1</sup>
H <sub>3</sub> BO <sub>3</sub>	2,91
MnCl <sub>2</sub> .4H <sub>2</sub> O	0,57
ZnSO <sub>4</sub> .7H <sub>2</sub> O	0,09
Na <sub>2</sub> MoO <sub>4</sub> .2H <sub>2</sub> O	0,03
CuSO <sub>4</sub> .5H <sub>2</sub> O	0,04
CoCl <sub>2</sub> .6H <sub>2</sub> O	0,05

D. Concentration of elements in medium

Element	μmole.l <sup>-1</sup>	μg.l <sup>-1</sup>
B	2,91	31,4
Mn	0,57	31,3
Zn	0,09	5,9
Mo	0,03	2,9
Cu	0,04	2,5
Co	0,05	2,9

(Total salt concentration = 244,6 mg.l<sup>-1</sup>)

Macronutrients were concentrated 1000-fold and made up separately in six stock solutions. All micronutrients were concentrated 1 000-fold and combined in a single stock solution.

with decreasing nutrient concentration. The highest batch-culture  $\overline{\text{RGR}}$  values of *A. filiculoides* in full-strength Azolla-I medium varied from 0,322 to 0,330  $\text{g.g}^{-1}.\text{day}^{-1}$  and corresponded to the highest rates obtained in these continuous cultures (0,325  $\text{g.g}^{-1}.\text{day}^{-1}$ ). The  $\overline{\text{RGR}}$  values obtained at low nutrient concentrations in batch-culture (Table 22) were increased by up to 52 % (from 0,167 to 0,254  $\text{g.g}^{-1}.\text{day}^{-1}$ ) when the plants were grown in continuous culture, due to the increased rate of nutrient supply, i.e. nutrient loading (Table 28). The range of loading rates (flow rate x concentration) used in this study covered a 128-fold increase in loading rates between the lowest and highest rates used. At low loading rates, those *A. filiculoides* plants growing at low nutrient concentrations (1/16 X) with high flow rates (4  $\text{l.hr}^{-1}$ ) had lower  $\overline{\text{RGR}}$  values than those plants grown with higher nutrient concentrations (1 X) and low flow rates (0,5  $\text{l.hr}^{-1}$ ). At moderate nutrient concentrations (1/4 X), an increase in flow rate above 1,0  $\text{l.hr}^{-1}$  did not significantly ( $P > 0,05$ ) increase  $\overline{\text{RGR}}$  values (Table 28). Therefore, the low flow rate of 1,0  $\text{l.hr}^{-1}$  was selected for further experiments with individual macronutrients.

TABLE 28 - The effects of flow rate and nutrient concentration on the  $\overline{\text{RGR}}^*$  of *A. filiculoides* ( $\text{g.g}^{-1}.\text{day}^{-1}$ ) in continuous culture. [Initial inoculum: 1,0 g. duration of experiment; 4 days (after 16 days acclimatization at 16L/8D, 45 kLux and 27 °C)]

Flow rate ( $\text{l.hr}^{-1}$ )	Medium concentration		
	1/16 X	1/4 X	1 X
0,5	0,167 $\pm$ 0,102	0,289 $\pm$ 0,116	0,324 $\pm$ 0,089
1,0	0,203 $\pm$ 0,107	0,301 $\pm$ 0,129	0,321 $\pm$ 0,114
2,0	0,228 $\pm$ 0,121	0,317 $\pm$ 0,140	0,325 $\pm$ 0,139
4,0	0,254 $\pm$ 0,118	0,321 $\pm$ 0,120	0,324 $\pm$ 0,096

[\* mean  $\pm$  two standard errors; n = 8]

A wide range of macronutrient concentrations was employed to determine the effects of nutrient concentration and the minimum level of each nutrient required to maintain high  $\overline{\text{RGR}}$  values in continuous culture (Table 29). In each series of experiments, the nutrient under investigation was supplied at four different concentrations while the other nutrients were maintained at full-strength. In the first series of experiments, the concentrations

TABLE 29 - The effects of different concentrations of macronutrients on the growth rate ( $\overline{\text{RGR}}$ ), nitrogenase activity (EPR) and nutrient content of *A. filiculoides* in continuous culture. [Flow rate: 1,0 l.hr<sup>-1</sup>; initial inoculum: 1,0 g; duration of experiment: 4 days (after 16 days acclimatization at 16L/8D, 45 kLux and 27 °C)]

Element	Conc. (mM.l <sup>-1</sup> )	Supply rate (µg.hr <sup>-1</sup> )	$\overline{\text{RGR}}^*$ (g.g <sup>-1</sup> .day <sup>-1</sup> )	EPR* nM(g.f.w.) <sup>-1</sup> .hr <sup>-1</sup>	% dry matter	% nutrient content in dry matter				
						N	Ca	Mg	K	P
Ca	0,0004	16	0,053 ± 0,018	315 ± 62	11,81	2,53	0,103	0,685	0,757	0,168
	0,004	160	0,127 ± 0,051	762 ± 71	8,20	3,63	0,168	0,672	0,752	0,227
	0,04	1600	0,320 ± 0,102	3010 ± 125	5,91	4,51	0,345	0,665	0,746	0,224
	0,4	16000	0,330 ± 0,117	3105 ± 158	5,86	4,65	0,450	0,668	0,759	0,231
Mg	0,0008	19	0,080 ± 0,022	492 ± 57	7,12	2,98	0,474	0,115	0,769	0,209
	0,008	190	0,169 ± 0,076	995 ± 86	6,50	3,77	0,455	0,448	0,758	0,218
	0,08	1900	0,318 ± 0,096	3090 ± 164	5,80	4,58	0,447	0,659	0,762	0,234
	0,8	19000	0,325 ± 0,115	3110 ± 151	5,85	4,59	0,449	0,673	0,763	0,229
K	0,0004	15	0,097 ± 0,023	554 ± 63	10,96	2,12	0,460	0,680	0,144	0,186
	0,004	150	0,240 ± 0,087	2005 ± 102	6,13	3,45	0,455	0,675	0,540	0,215
	0,04	1500	0,329 ± 0,098	3155 ± 127	5,83	4,63	0,441	0,670	0,759	0,224
	0,4	15000	0,328 ± 0,096	3125 ± 186	5,83	4,55	0,446	0,672	0,764	0,220
P	0,0004	12,4	0,147 ± 0,051	940 ± 87	9,65	2,04	0,465	0,681	0,748	0,073
	0,004	124	0,321 ± 0,095	3015 ± 141	5,86	4,48	0,440	0,670	0,737	0,149
	0,04	1240	0,324 ± 0,087	3100 ± 203	5,83	4,61	0,443	0,668	0,749	0,196
	0,4	12400	0,316 ± 0,102	3085 ± 129	5,84	4,56	0,449	0,676	0,752	0,230

[\* Mean ± two standard errors]

of each nutrient under test were varied over three orders of magnitude to identify the approximate range of the threshold level of each nutrient (Table 29). A second series of experiments was then carried out over a much smaller concentration range accurately<sup>†</sup> locate the threshold or minimum nutrient concentration required for optimum growth (Table 30).

For the four macronutrients investigated (Ca, Mg, K and P), the first series of experiments clearly demonstrated the approximate range of the minimum concentration required (Table 29). When *A. filiculoides* plants were grown at sub-optimal concentrations of a particular nutrient, the content of that nutrient within the plants decreased as did the nitrogenase activity and  $\overline{\text{RGR}}$  values. Plants of *A. filiculoides* grown in sub-optimal concentrations of Ca, Mg and K also exhibited a decrease in P content. Similar trends were also recorded in the second series of experiments though they were not as marked due to the smaller concentration range used (Table 30).

From the above experiments the threshold levels of Ca, Mg, K and P could be estimated. These were estimated as the minimum concentration of each nutrient required to maintain nitrogenase activity and  $\overline{\text{RGR}}$  values at optimum levels (Yatazawa *et al.*, 1980). The threshold levels of Ca, Mg, K and P for optimum  $\overline{\text{RGR}}$  of *A. filiculoides* were found to be 0,3, 0,6, 0,03 and 0,004  $\text{mmoles.l}^{-1}$  respectively. The threshold levels of these elements for full development of nitrogenase activity were 0,2, 0,4, 0,03 and 0,004  $\text{mmoles.l}^{-1}$  respectively. When nutrients were supplied at concentrations below their respective threshold levels, they caused a decrease in nitrogenase activity and  $\overline{\text{RGR}}$  values (Table 30).

At optimum and supra-optimum nutrient concentrations, the nutrient content of *A. filiculoides* plants remained relatively stable and, with the exception of P, did not continue to increase with increasing nutrient concentration (Tables 29 and 30). At these high rates of nutrient supply, the N, Ca, Mg and K contents of the plants averaged 4,62 %, 0,446 %, 0,671 % and 0,760 % of the dry mass respectively.

In the field, crude estimates of flow rate were made using floating drift cards. Flow rates at the nine riverine stations varied from 7 200 to

TABLE 30 - The effect of different levels of Ca, Mg, K and P on the mean relative growth rate ( $\overline{\text{RGR}}$ ), nitrogenase activity (EPR), % dry matter and nutrient content of *A. filiculoides* in continuous culture. [Flow rate: 1,0 l.hr<sup>-1</sup>; initial inoculum: 1,0 g; duration of experiment: 4 days (after 16 days acclimatization at 16L/8D, 45 kLux and 27 °C)]

Element	Conc. (mM.l <sup>-1</sup> )	Supply rate (µg.hr <sup>-1</sup> )	$\overline{\text{RGR}}^*$ (g.g <sup>-1</sup> .day <sup>-1</sup> )	EPR* nM(g.f.w.) <sup>-1</sup> .hr <sup>-1</sup>	% dry matter	% nutrient content in dry matter				
						N	Ca	Mg	K	P
Ca	0,1	4000	0,320 ± 0,099	3097 ± 81	5,91	4,59	0,397	0,665	0,749	0,227
	0,2	8000	0,328 ± 0,126	3184 ± 138	5,88	4,64	0,429	0,667	0,763	0,228
	0,3	12000	0,335 ± 0,087	3250 ± 116	5,84	4,70	0,446	0,666	0,771	0,231
	0,4	16000	0,336 ± 0,109	3146 ± 155	5,87	4,67	0,444	0,670	0,766	0,230
Mg	0,2	4876	0,320 ± 0,142	3098 ± 117	5,83	4,61	0,450	0,664	0,761	0,234
	0,4	9752	0,326 ± 0,099	3169 ± 149	5,82	4,65	0,444	0,662	0,767	0,234
	0,6	14628	0,338 ± 0,115	3220 ± 94	5,87	4,69	0,448	0,671	0,763	0,235
	0,8	19504	0,331 ± 0,128	3190 ± 246	5,85	4,62	0,451	0,669	0,769	0,238
K	0,01	390	0,287 ± 0,098	2680 ± 102	6,07	3,95	0,449	0,679	0,668	0,228
	0,02	780	0,302 ± 0,141	2874 ± 89	5,94	4,24	0,441	0,672	0,747	0,237
	0,03	1170	0,340 ± 0,102	3198 ± 168	5,89	4,60	0,442	0,675	0,771	0,236
	0,04	1560	0,333 ± 0,117	3121 ± 138	5,84	4,65	0,447	0,674	0,772	0,234
P	0,001	31	0,192 ± 0,072	1648 ± 79	6,88	2,83	0,460	0,679	0,748	0,115
	0,002	62	0,257 ± 0,089	2315 ± 152	6,34	3,61	0,452	0,670	0,750	0,131
	0,003	93	0,303 ± 0,098	2863 ± 140	5,96	4,28	0,440	0,662	0,750	0,140
	0,004	124	0,328 ± 0,125	3127 ± 176	5,87	4,59	0,442	0,663	0,754	0,152

[\* Mean ± two standard errors]

54000  $\ell \cdot \text{hr}^{-1}$  in winter and 180000 to 720000  $\ell \cdot \text{hr}^{-1}$  in summer. The very high flow rates in summer were recorded during the annual floods in January each year.

#### 6.3.5 Nutrient deficiencies and ion interactions

The  $\overline{\text{RGR}}$  and nitrogenase activity of *A. filiculoides* grown in macronutrient deficient media for 32 days were compared to those of plants grown in complete (nitrogen-free) Azolla-I medium (Table 31). All nutrient deficiencies caused a reduction in  $\overline{\text{RGR}}$  and nitrogenase activity. Calcium-deficient media caused the greatest decreases (96 and 99 % respectively) whilst the smallest reductions were recorded in sulphur-deficient media (34 and 42 % respectively). Media deficient in Mg, K, P and Fe had similar effects, reducing  $\overline{\text{RGR}}$  values by 79 to 85 % and nitrogenase activity by 91 to 96 %.

In order to determine whether nutrient deficiencies had differential effects on  $\overline{\text{RGR}}$  and nitrogenase activity, the "condition ratio" of Yatazawa *et al.* (1980) was also calculated. This ratio equals unity when both processes are equally affected, and is greater than one or less than one depending on whether one of the processes ( $\overline{\text{RGR}}$ ) is reduced or increased relative to the other (nitrogenase activity). For all *A. filiculoides* plants grown in macronutrient-deficient media, the condition ratio indicated that the decrease in nitrogenase activity was greater than the reduction in  $\overline{\text{RGR}}$  values (Table 31). Here, calcium-deficient plants were most severely affected and sulphur-deficient plants the least affected.

The following characteristic deficiency symptoms were observed when plants of *A. filiculoides* were grown in nutrient deficient culture solutions:

Calcium	Foliar symptoms were first visible after four days, the leaves becoming initially chlorotic followed by the development of large (1 mm diameter) necrotic spots on the dorsal leaf lobes, and the plants died within 35 days.
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TABLE 31 - Changes in the growth rate ( $\overline{\text{RGR}}$ :  $\text{g.g}^{-1}.\text{day}^{-1}$ ), nitrogenase activity (EPR:  $\text{nM. (g.f.wt)}^{-1}.\text{hr}^{-1}$ ) and nitrogen content of *A. filiculoides* cultured on macro-deficient media for 32 days

Medium	$\overline{\text{RGR}}^*$	% of control (A)	EPR*	% of control (B)	% N	Condition ratio (B)/(A)
Complete	0,341 $\pm$ 0,072	100	3150 $\pm$ 127	100	4,57	1,000
-Ca	0,013 $\pm$ 0,004	4,1	18 $\pm$ 5	0,6	1,42	0,146
-Mg	0,065 $\pm$ 0,012	20,7	130 $\pm$ 27	4,2	2,06	0,203
-K	0,050 $\pm$ 0,011	15,9	276 $\pm$ 54	8,9	2,34	0,560
-P	0,048 $\pm$ 0,009	15,3	170 $\pm$ 26	5,5	2,15	0,359
-S	0,208 $\pm$ 0,068	66,2	1809 $\pm$ 96	58,3	3,97	0,881
-Fe	0,052 $\pm$ 0,007	16,3	166 $\pm$ 18	5,3	2,28	0,325

[\*mean  $\pm$  two standard errors; n = 6]

TABLE 32 - Changes in the growth rate ( $\overline{\text{RGR}}$ :  $\text{g.g}^{-1}.\text{day}^{-1}$ ), nitrogenase activity (EPR:  $\text{nM. (g.f.wt)}^{-1}.\text{hr}^{-1}$ ) and nitrogen content of *A. filiculoides* cultured on micronutrient-deficient media for 48 days

Medium	$\overline{\text{RGR}}^*$	% of control (A)	EPR*	% of control (B)	% N	Condition ratio (B)/(A)
Complete	0,316 $\pm$ 0,079	100	3124 $\pm$ 131	100	4,57	1,000
-Mn	0,171 $\pm$ 0,077	54,2	1106 $\pm$ 55	35,4	2,68	0,653
-Zn	0,235 $\pm$ 0,099	74,4	1749 $\pm$ 114	56,0	3,31	0,753
-Mo	0,193 $\pm$ 0,085	61,1	961 $\pm$ 71	30,8	2,82	0,504
-Cu	0,253 $\pm$ 0,107	80,1	1983 $\pm$ 104	63,5	3,62	0,793
-Co	0,241 $\pm$ 0,092	76,3	1478 $\pm$ 113	47,3	3,03	0,620
-B	0,103 $\pm$ 0,011	32,6	1143 $\pm$ 52	36,6	2,74	1,123

[\*mean  $\pm$  two standard errors; n = 6]

Magnesium	Foliar symptoms were first visible after eight days, the leaves initially turned a pale yellow colour, later becoming very chlorotic with small (0,2 to 0,5 mm diameter) necrotic spots on the dorsal leaf lobes. Plants became very brittle and fragmented easily after 12 days and all plants had died within 35 days.
Potassium	Foliar symptoms were first visible after eight days and were identical to those of the magnesium-deficient plants. Plants died within 30 days.
Phosphorus	Foliar symptoms were visible after six days, the leaf lobes developed a deep reddish-brown colouration with chlorotic leaf lobe margins. Roots became very elongated and curled. Plants died within 30 days.
Iron	Foliar symptoms only observed after eight days, and closely resembled the symptoms of magnesium-deficient plants. Plants died within 40 days.
Sulphur	Foliar symptoms were first visible after 12 days, the centre of the dorsal leaf lobe turned a dark green colour while the leaf lobe margins were very pale yellow and chlorotic. All plants were still alive after 50 days of culture.

The effects of micronutrient deficiencies on rates of growth and nitrogenase activity took very much longer to become manifested and necessitated the continuation of these experiments for 48 days. These results are shown in Table 32.  $\overline{\text{RGR}}$  values were highest in copper- and cobalt-deficient media and lowest in boron- and manganese-deficient media. Deficiencies of Mo, Mn and B caused the greatest reductions in nitrogenase activity. The condition ratio data suggested that Mo, Co and Mn deficiencies affected the development of nitrogenase activity while B affected  $\overline{\text{RGR}}$  specifically. No definite foliar symptoms of micronutrient deficiency could be distinguished in this series of experiments. All the plants were similar in appearance, light yellowish-green with pale yellow leaf lobe margins.

The results of nutrient triangle experiments to investigate the interacting effects of nutrients supplied at sub-optimal concentrations and the effects of media deficient in either one or two nutrients are shown in Table 33 (cations) and Table 34 (anions). The media with single nutrient de-

TABLE 33 - Cation triangle experiment. The effects of different proportions of cations on the growth rate ( $\overline{\text{RGR}}$ :  $\text{g.g}^{-1}.\text{day}^{-1}$ ), nitrogenase activity (EPR:  $\text{nM. (g.f.wt)}^{-1}.\text{hr}^{-1}$ ) and nitrogen content of *A. filiculoides*. [Cation ratios are expressed in one-eighths of the final mixture (Section 6.2.4)]

Treat- ment No.	Cation ratio K-Ca-Mg	$\overline{\text{RGR}}^*$	% of control (A)	EPR*	% of control (B)	% N	Condition ratio (B)/(A)
1	8 0 0	0,011 $\pm$ 0,002	3,5	12 $\pm$ 5	0,4	1,29	0,114
2	6 1 1	0,078 $\pm$ 0,014	24,8	270 $\pm$ 41	8,7	2,34	0,351
3	5 2 1	0,093 $\pm$ 0,021	29,5	501 $\pm$ 23	16,1	2,52	0,546
4	5 1 2	0,086 $\pm$ 0,019	27,3	393 $\pm$ 24	12,6	2,50	0,462
5	4 4 0	0,062 $\pm$ 0,012	19,7	103 $\pm$ 26	3,3	1,94	0,167
6	4 2 2	0,203 $\pm$ 0,067	64,4	2053 $\pm$ 113	65,9	3,97	1,023
7	4 0 4	0,015 $\pm$ 0,003	4,8	26 $\pm$ 7	0,8	1,38	0,167
8	3 3 2	0,242 $\pm$ 0,080	76,8	2545 $\pm$ 129	81,7	4,16	1,064
9	3 2 3	0,211 $\pm$ 0,051	67,0	2159 $\pm$ 142	69,3	4,01	1,034
10	2 5 1	0,168 $\pm$ 0,038	53,3	1663 $\pm$ 97	53,4	3,64	1,002
11	2 4 2	0,246 $\pm$ 0,052	78,1	2670 $\pm$ 148	85,7	4,21	1,097
12	2 3 3	0,214 $\pm$ 0,058	67,9	2237 $\pm$ 125	71,8	4,09	1,057
13	2 2 4	0,150 $\pm$ 0,029	47,6	1232 $\pm$ 103	39,6	3,22	0,832
14	2 1 5	0,124 $\pm$ 0,027	39,4	887 $\pm$ 68	28,5	2,80	0,723
15	1 6 1	0,137 $\pm$ 0,038	43,5	1084 $\pm$ 101	34,8	3,07	0,800
16	1 5 2	0,174 $\pm$ 0,047	55,2	1737 $\pm$ 109	55,8	3,85	1,011
17	1 2 5	0,131 $\pm$ 0,034	41,6	989 $\pm$ 87	31,7	2,91	0,762
18	1 1 6	0,126 $\pm$ 0,027	40,0	810 $\pm$ 42	26,0	2,42	0,650
19	0 8 0	0,019 $\pm$ 0,004	6,0	52 $\pm$ 17	1,7	1,89	0,283
20	0 4 4	0,053 $\pm$ 0,013	16,8	147 $\pm$ 23	4,7	2,12	0,280
21	0 0 8	0,009 $\pm$ 0,002	2,9	11 $\pm$ 6	0,4	1,37	0,138

[\*mean  $\pm$  two standard errors; n = 8]

TABLE 34 - Anion triangle experiment. The effects of different proportions of anions on the growth rate ( $\overline{\text{RGR}}$ :  $\text{g.g}^{-1}.\text{day}^{-1}$ ), nitrogenase activity (EPR:  $\text{nM.}(\text{g.f.wt})^{-1}.\text{hr}^{-1}$ ) and nitrogen content of *A. filiculoides*. [Anion ratios are expressed in one-eighths of the final mixture (Section 6.2.4)]

Treat- ment No.	Anion ratio P-Cl-S	$\overline{\text{RGR}}^*$	% of control (A)	EPR*	% of control (B)	% N	Condition ratio (B)/(A)
22	8 0 0	0,183 $\pm$ 0,039	58,1	764 $\pm$ 92	24,5	2,86	0,422
23	6 1 1	0,208 $\pm$ 0,048	66,0	1173 $\pm$ 125	37,7	3,24	0,571
24	5 2 1	0,210 $\pm$ 0,050	66,7	1298 $\pm$ 166	41,7	3,34	0,625
25	5 1 2	0,233 $\pm$ 0,067	74,0	1440 $\pm$ 141	46,2	3,44	0,624
26	4 4 0	0,152 $\pm$ 0,031	48,3	1165 $\pm$ 98	37,4	3,29	0,774
27	4 2 2	0,285 $\pm$ 0,055	90,5	2580 $\pm$ 156	82,8	4,35	0,915
28	4 0 4	0,164 $\pm$ 0,032	52,1	1329 $\pm$ 87	42,7	3,37	0,820
29	3 3 2	0,288 $\pm$ 0,062	91,4	2623 $\pm$ 182	84,2	4,36	0,921
30	3 2 3	0,271 $\pm$ 0,048	86,0	2393 $\pm$ 103	76,8	4,21	0,893
31	2 5 1	0,196 $\pm$ 0,036	62,2	1265 $\pm$ 85	40,6	3,32	0,653
32	2 4 2	0,240 $\pm$ 0,061	76,2	1751 $\pm$ 122	56,2	3,86	0,738
33	2 3 3	0,251 $\pm$ 0,043	79,7	1909 $\pm$ 163	61,3	4,01	0,769
34	2 2 4	0,262 $\pm$ 0,057	83,2	2167 $\pm$ 177	69,6	4,08	0,837
35	2 1 5	0,203 $\pm$ 0,052	64,4	1704 $\pm$ 75	54,7	3,83	0,849
36	1 6 1	0,184 $\pm$ 0,041	58,4	1115 $\pm$ 128	35,8	3,19	0,613
37	1 5 2	0,207 $\pm$ 0,046	65,7	1559 $\pm$ 124	50,1	3,60	0,763
38	1 2 5	0,209 $\pm$ 0,057	66,3	1593 $\pm$ 116	51,1	3,64	0,771
39	1 1 6	0,206 $\pm$ 0,049	65,4	1598 $\pm$ 146	51,3	3,61	0,784
40	0 8 0	0,045 $\pm$ 0,013	14,3	69 $\pm$ 14	2,2	1,96	0,154
41	0 4 4	0,042 $\pm$ 0,011	13,3	81 $\pm$ 17	2,6	2,08	0,195
42	0 0 8	0,039 $\pm$ 0,011	12,4	43 $\pm$ 10	1,4	2,02	0,113

[\*mean  $\pm$  two standard errors; n = 8]

ficiencies of Mg, Ca and K (treatments 5, 7 and 20 respectively) reduced values of  $\overline{\text{RGR}}$  and nitrogenase activity to levels similar to those shown in Table 31. Media deficient in two nutrients (treatments 1, 19 and 21 respectively) resulted in even greater reductions in  $\overline{\text{RGR}}$  and nitrogenase activity (Table 33). Higher values of  $\overline{\text{RGR}}$  and nitrogenase activity were recorded in treatments 8 and 11. At the intermediate treatments (6, 8, 9, 10, 11, 12 and 16), located near the centre of the triangle (Figure 55), the condition ratios were all greater than unity, indicating a greater reduction in  $\overline{\text{RGR}}$  than nitrogenase activity. The position of these treatments within the triangle (Figure 55) indicated that a decrease in Ca concentration could not be compensated for by increased Mg levels and that high levels of K could only partly compensate for decreased Ca levels. In the triangle of anion treatments (Table 34), deficiencies of both Cl and  $\text{SO}_4$  caused only slight reduction in  $\overline{\text{RGR}}$  and nitrogenase activity, while P deficiency greatly reduced  $\overline{\text{RGR}}$  and nitrogenase activity values. The media deficient in P and either Cl or  $\text{SO}_4$  resulted in an even greater reduction in both  $\overline{\text{RGR}}$  and nitrogenase activity. In the remainder of the treatments,  $\overline{\text{RGR}}$  and nitrogenase activity values were relatively high, though the condition ratios indicated a greater reduction in nitrogenase activity relative to  $\overline{\text{RGR}}$ .

#### 6.3.6 Limiting nutrients in the natural environment

The chemical characteristics of water from beneath a multi-layered *A. filiculoides* mat were clearly different to those from open water areas (Table 35). Nutrient concentrations in the open water at the riverine site (station 10) were lower than nutrient concentrations from the open water at station 9, the farm dam. In addition, nutrient concentrations in the water beneath *Azolla* mats were considerably higher than concentrations in the open water at both stations. On average, major cations in water beneath the *Azolla* mats were from 5 to 27 % higher and minor cations were 29 to 300 % higher than the open water situation. The presence of an *Azolla* mat also resulted in higher concentrations of  $\text{PO}_4\text{-P}$  (90 to 130 %) and inorganic nitrogen (7 to 107 %). The relatively high  $\text{NO}_3\text{-N}$  concentrations found beneath the *A. filiculoides* mats reflect the conditions in the water immediately below the mat, which was usually aerobic. The deeper waters were most often anaerobic and consequently had very low levels of  $\text{NO}_3\text{-N}$ . However, compared with the concentration of nutrients in full-strength *Azolla*-I medium, the concentrations of the macronutrients Ca, Mg, Na, K and P in water beneath

an *Azolla* mat were always 14 to 85 % lower than the Azolla-I medium. In contrast, the concentrations of all micronutrients except Fe, B and Mo were between 1 and 15 times greater than their concentrations in Azolla-I medium. The concentrations of these elements were respectively 84 %, 80 % and 40 % lower than the optimum level in Azolla-I medium. Consequently, in terms of the concentrations of nutrients required for maximum  $\overline{\text{RGR}}$  and nitrogenase activity, these analyses suggested that the macronutrients Ca, Mg, Na, K and P, and the micronutrients Fe, B and Mo were at sub-optimal concentrations and therefore likely to be limiting to the growth of *A. filiculoides*.

TABLE 35 - Chemical characteristics ( $\text{mg.}\ell^{-1}$ ) of water samples collected from the open water and from beneath multi-layered mats of *A. filiculoides* at a farm dam (station 9) and a riverine site (station 10). [Each result is a mean of 4 composite samples]

Parameter	Station 9			Station 10		
	Open water	Under mat	% Incr.	Open water	Under mat	% Incr.
Ca	10,73	12,12	13 %	9,98	10,86	9 %
Mg	13,51	16,20	9 %	12,98	15,09	16 %
K	2,26	2,38	5 %	2,21	2,35	6 %
Na	7,46	9,10	16 %	6,97	8,88	27 %
Fe	0,065	0,155	138 %	0,055	0,095	73 %
Mn	0,110	0,155	41 %	0,045	0,075	67 %
Zn	0,035	0,035	0	0,025	0,025	0
Cu	0,035	0,045	29 %	0,025	0,025	80 %
Co	0,001	0,004	300 %	0,001	0,002	100 %
Mo	0,001	0,002	100 %	0,001	0,002	100 %
B	0,002	0,006	300 %	0,002	0,004	100 %
Cl	17,69	20,48	16 %	15,13	16,91	12 %
SO <sub>4</sub>	21,26	26,95	27 %	13,73	16,05	17 %
Total alkalinity	31,54	34,51	9 %	45,52	53,56	18 %
PO <sub>4</sub> <sup>-P</sup>	0,91	1,73	90 %	0,16	0,37	131 %
NH <sub>4</sub> <sup>-N</sup>	0,43	0,63	47 %	0,28	0,30	7 %
NO <sub>2</sub> <sup>-N</sup> + NO <sub>3</sub> <sup>-N</sup>	1,68	3,48	107,4 %	1,10	1,23	12 %
pH	9,21	9,03		9,22	9,15	

This hypothesis was tested by conducting a series of growth trials in the field, using nutrient-spiked water samples collected from both the open water and beneath an *Azolla* mat. Each of the nutrients thought to be limiting was tested singly and then in combinations in samples of open water and "mat water" at both sampling stations. The results of these trials are summarized in Table 36.

*Azolla filiculoides* plants at both stations 9 and 10 gave similar responses to nutrient additions (Table 36). On the whole, the increases in  $\overline{\text{RGR}}$  and nitrogenase activity at station 10 (the riverine site, which originally had lower concentrations of nutrients) relative to the control, were slightly higher than those recorded at station 9. With the exception of B, all nutrient spikes caused almost equal increases in  $\overline{\text{RGR}}$  and nitrogenase activity. In those media spiked with B, the increase in  $\overline{\text{RGR}}$  values was almost twice the increase in nitrogenase activity (Table 36). Single nutrient spikes of P or Fe caused the largest increases in  $\overline{\text{RGR}}$  values (10,3 to 16,7 %) and nitrogenase activity (12,8 to 18,5 %) respectively, whilst nutrient spikes with Mg, K and Na gave lower increases in  $\overline{\text{RGR}}$  values (2,8 to 4,3 %) and nitrogenase activity (2,7 to 4,1 %). Intermediate increases in  $\overline{\text{RGR}}$  and nitrogenase activity were obtained with spikes of Ca, Mo and B.

When water samples were enriched with combinations of nutrients, higher values of  $\overline{\text{RGR}}$  and nitrogenase activity were recorded (Table 36). Addition of Ca, P and Fe in combination, increased  $\overline{\text{RGR}}$  values by 26,7 to 34,0 % and nitrogenase activity by 28,5 to 35,1 % relative to the control. An additional combination, containing the above three nutrients plus Mo and B, further increased  $\overline{\text{RGR}}$  and nitrogenase activity by 1,5 to 4,1 %. Again, the greatest increases were recorded in water from station 10, the riverine site.

#### 6.3.7 Release of nutrients by decaying *A. filiculoides* plants

The investigation of the rates of nutrient loss from desiccated and live *A. filiculoides* material was carried out during October 1973. To facilitate discussion, the terms "desiccated" and "live" were used to describe the two respective sets of material.

TABLE 36 - The growth rates ( $\overline{\text{RGR}}^*$ :  $\text{g.g}^{-1}.\text{day}^{-1}$ ) and nitrogenase activity ( $\text{EPR}^{**}$ :  $\text{nM.}(\text{g.f.wt})^{-1}.\text{hr}^{-1}$ ) of *A. filiculoides* grown *in situ* at stations 9 and 10 in samples of water from beneath an *Azolla* mat and the open water after single or multiple nutrient enrichments. [Figures in brackets indicate percentage increase relative to control]

Treatment	Conc. supplied	Station 9				Station 10			
		Open water		Under mat		Open water		Under mat	
		$\overline{\text{RGR}}$	EPR	$\overline{\text{RGR}}$	EPR	$\overline{\text{RGR}}$	EPR	$\overline{\text{RGR}}$	EPR
Control	(no addition)	0,178	1868	0,105	2060	0,162	1784	0,178	1913
+Ca	10 $\text{mg.l}^{-1}$	0,193 (8,4 %)	2038 (9,1 %)	0,209 (7,2 %)	2212 (7,4 %)	0,177 (9,3 %)	1952 (9,4 %)	0,193 (8,4 %)	2077 (8,6 %)
+Mg	10 $\text{mg.l}^{-1}$	0,184 (3,4 %)	1932 (3,4 %)	0,201 (3,1 %)	2122 (3,0 %)	0,169 (4,3 %)	1857 (4,1 %)	0,184 (3,4 %)	2022 (3,5 %)
+K	10 $\text{mg.l}^{-1}$	0,183 (2,9 %)	1920 (2,8 %)	0,200 (3,0 %)	2116 (2,7 %)	0,168 (3,7 %)	1846 (3,5 %)	0,184 (2,9 %)	1971 (3,0 %)
+Na	20 $\text{mg.l}^{-1}$	0,183 (2,9 %)	1918 (2,7 %)	0,199 (2,8 %)	2115 (2,7 %)	0,167 (3,1 %)	1849 (3,6 %)	0,183 (2,8 %)	2021 (3,5 %)
+P	5 $\text{mg.l}^{-1}$	0,204 (14,1 %)	2176 (16,5 %)	0,218 (11,8 %)	2328 (13,0 %)	0,189 (16,7 %)	2114 (18,5 %)	0,206 (15,7 %)	2250 (17,6 %)
+Fe	1 $\text{mg.l}^{-1}$	0,199 (11,8 %)	2133 (14,2 %)	0,215 (10,3 %)	2320 (12,9 %)	0,181 (11,7 %)	2025 (13,5 %)	0,197 (10,7 %)	2158 (12,8 %)
+Mo	2 $\mu\text{g.l}^{-1}$	0,191 (7,3 %)	2025 (8,4 %)	0,208 (6,7 %)	2225 (8,0 %)	0,176 (8,6 %)	1946 (9,1 %)	0,190 (6,7 %)	2062 (7,8 %)
+B	20 $\mu\text{g.l}^{-1}$	0,192 (7,9 %)	1941 (3,9 %)	0,208 (6,7 %)	2132 (3,5 %)	0,175 (8,0 %)	1880 (5,4 %)	0,190 (6,7 %)	2003 (4,7 %)
+Ca+P+Fe		0,228 (28,1 %)	2460 (31,7 %)	0,247 (26,7 %)	2647 (28,5 %)	0,217 (34,0 %)	2410 (35,1 %)	0,235 (32,0 %)	1913 (33,0 %)
+Ca+P+Fe+Mo+B		0,233 (30,9 %)	2507 (34,2 %)	0,250 (28,2 %)	2679 (30,1 %)	0,223 (37,7 %)	2478 (38,9 %)	0,242 (35,9 %)	2622 (37,1 %)

[\*RGR measured from day 14 to day 21; \*\*EPR measured on day 21]

Live plants showed a slight (2 to 4 %) reduction in mass during the first 48 hours (Figure 56) after which loss in biomass occurred at a constant rate of approximately  $3,3 \text{ \%} \cdot \text{day}^{-1}$  (Figure 57). After 30 days, only 8,2 % of the original plant mass remained in the litter bag. Desiccated plant material showed an initial loss of 15 % in 2 hours (Figure 56), but this loss rate decreased after 4 hours and thereafter remained more or less constant at  $2,03 \text{ \%} \cdot \text{day}^{-1}$  (Figure 57). After 30 days, 28,7 % of the original plant mass remained, a significantly higher ( $P < 0,01$ ) proportion than the bags containing live plant material.

The nutrient content of desiccated and live *A. filiculoides* material prior to the experiment was almost identical, with the exception that the desiccated plants contained approximately 10 % less N. The nutrient proportions of the undecomposed live material remaining within the litter bags showed some variation during the 30 day experiment (Table 37). The proportions of N, K and Ca remained approximately constant, whilst P, Mg and Fe decreased approximately linearly by 20 %, 29 % and 42 % respectively over the 30 day experiment.

The proportions of nutrients within the remaining undecomposed desiccated material were, on the whole, quite different from the live material (Table 37). The proportions of Ca and Mg increased by 61 % and 63 % respectively during the first day and thereafter Ca remained approximately constant while the Mg content slowly declined. The K content of the desiccated material decreased by 91 % in the first day and then remained constant. The proportions of P and Fe decreased by 38 % and 45 % respectively during the first day, followed by a more gradual decrease of approximately  $1 \text{ \%} \cdot \text{day}^{-1}$  to a value of 0,08 % after 30 days (Table 37). Throughout the study, the N content of the desiccated *A. filiculoides* material remained approximately constant.

The different rates of nutrient loss (Table 37) resulted in marked differences between the total nutrient stocks remaining in decomposing live and desiccated *A. filiculoides* material (Figure 58). Stocks of N, P, K and Fe in desiccated material decreased by 12, 55, 92 and 44 % respectively within the first day (Figure 58a, b, c, and f). Thereafter, no further loss in K was recorded whilst the N, P and Fe stocks were reduced

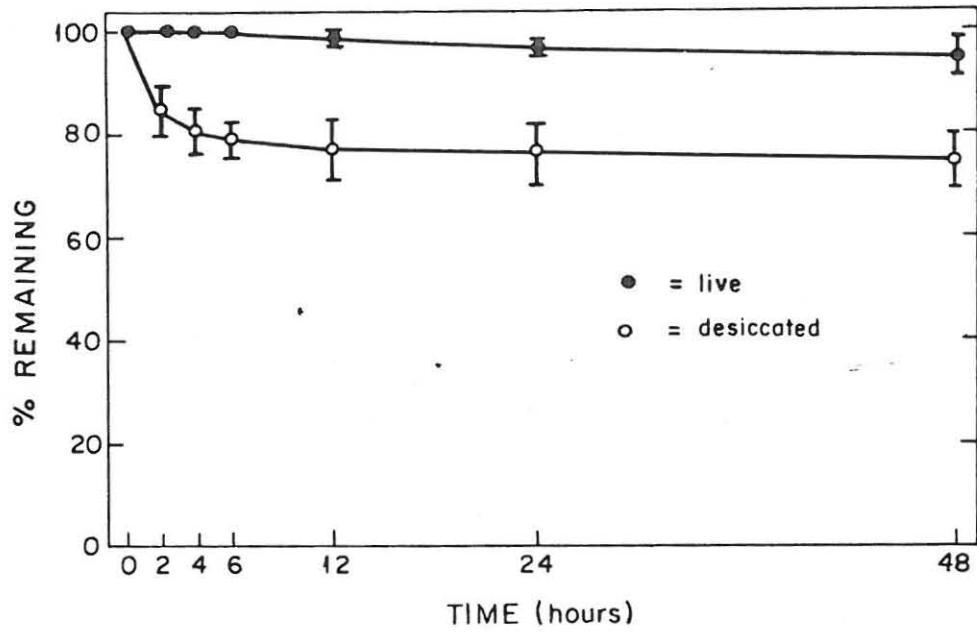


FIG. 56 *Initial loss of dry weight during decomposition of desiccated and live A. filiculoides plants. (Each value is a mean of four samples). [Vertical bars indicate two standard errors].*

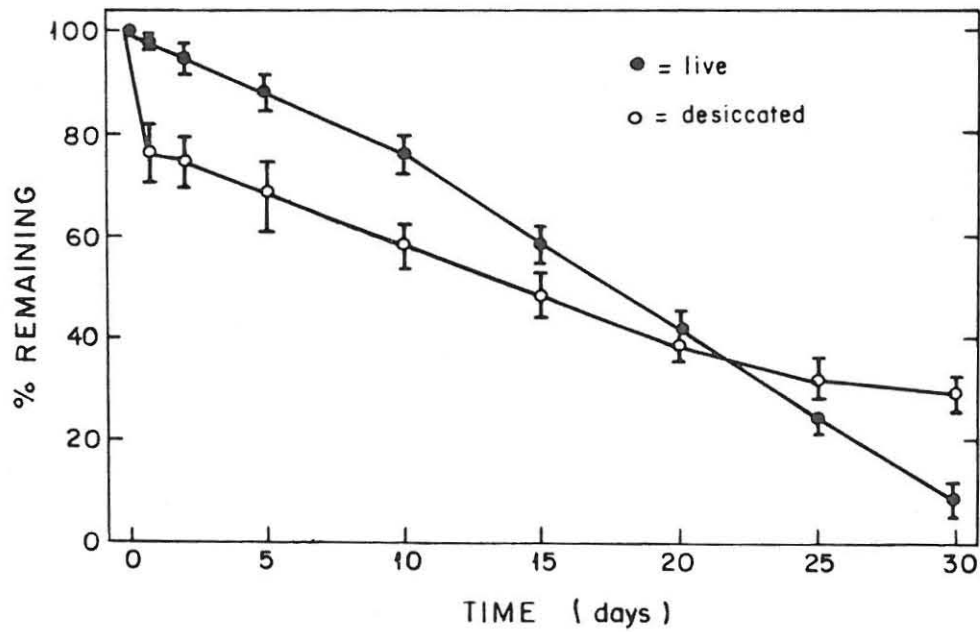


FIG. 57 *Total loss of dry weight during decomposition of desiccated and live A. filiculoides plants. (Each value is a mean of four samples). [Vertical bars indicate two standard errors].*

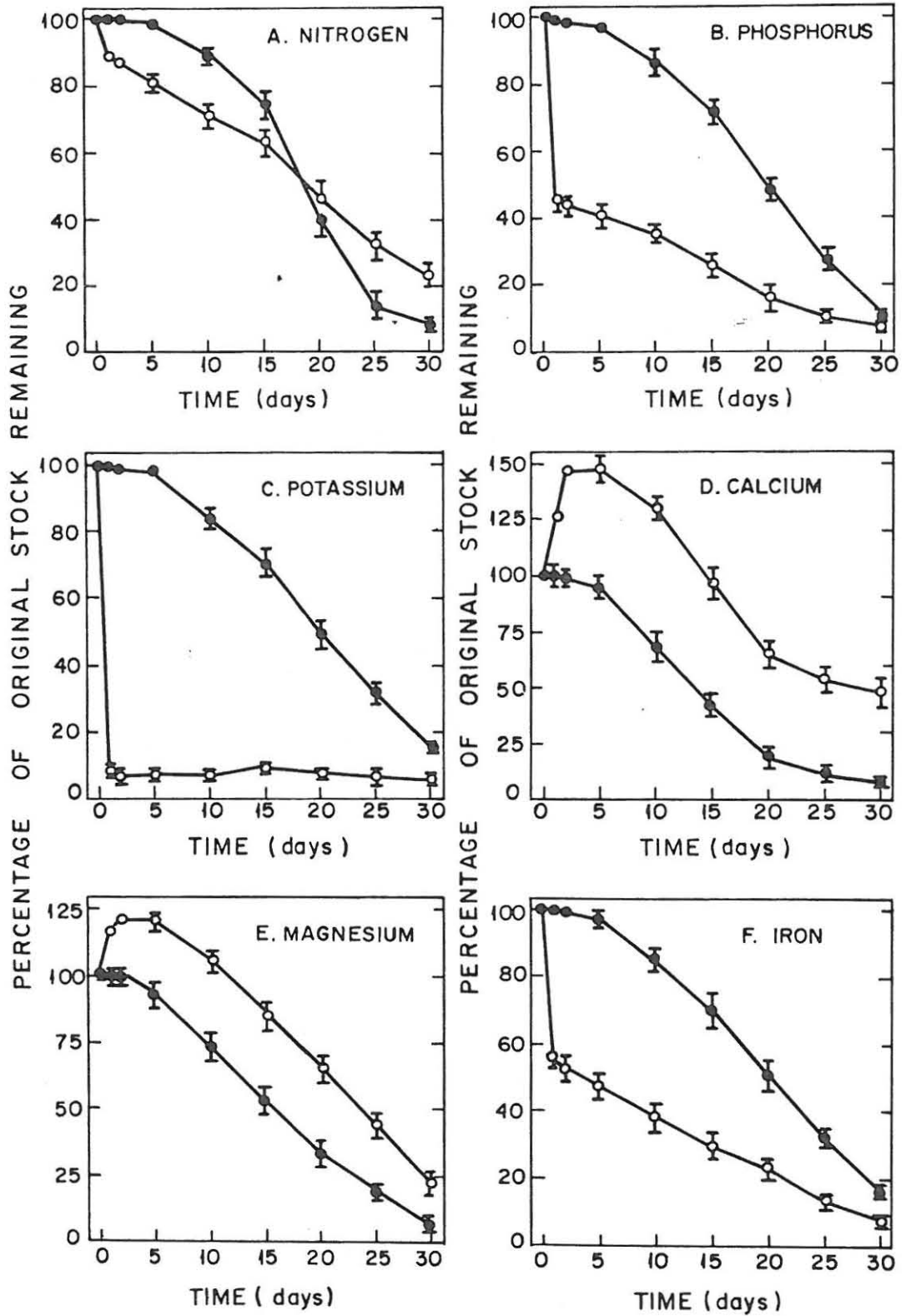


FIG. 58 *Changes in nutrient content of live (●—●) and desiccated (○—○) A. filiculoides during decomposition. (Each value is a mean of four samples). [Vertical bars indicate two standard errors].*

TABLE 37 - Levels of mineral elements in desiccated and live *A. filiculoides* plants initially, and during decomposition below an *Azolla* mat. (All values as percentage of dry weight)

Plant material	Analysis (%)	Time (days from start of decomposition)				
		0	1	10	20	30
Live	N	4,15	4,17	4,13	4,14	4,12
	P	0,20	0,21	0,19	0,18	0,16
	K	0,96	0,98	0,95	0,94	0,95
	Ca	0,72	0,71	0,74	0,70	0,72
	Mg	0,68	0,68	0,62	0,55	0,48
	Fe	0,19	0,18	0,16	0,14	0,11
Desiccated	N	3,78	3,75	3,80	3,74	3,75
	P	0,21	0,13	0,11	0,09	0,08
	K	0,94	0,08	0,07	0,08	0,07
	Ca	0,70	1,13	1,13	1,10	1,11
	Mg	0,67	1,09	1,05	0,99	0,94
	Fe	0,20	0,11	0,10	0,09	0,08

at nearly constant rates of 2,24, 1,62 and 1,66  $\% \cdot \text{day}^{-1}$  respectively. The stocks of Ca and Mg in desiccated material increased by 46 % and 18 % respectively during the first day, but this increase was followed by a short stable period of 4 days and then both elements were lost at an almost constant rate of 4  $\% \cdot \text{day}^{-1}$ .

Nutrient loss from live *A. filiculoides* material was characterized by an initial short lag period of approximately 5 days, after which all nutrients were lost at virtually constant rates. These rates of nutrient loss varied from 3,2  $\% \cdot \text{day}^{-1}$  for Fe to 3,7  $\% \cdot \text{day}^{-1}$  for N.

#### 6.3.8 The influence of pH on nutrient uptake

The concentration of Ca and the form in which Fe was supplied in the nutrient medium affected the  $\overline{\text{RGR}}$  and nitrogenase activity of *A. filiculoides* over a wide range of culture pH (Table 38). At most of the PH levels and Fe sources tested,  $\overline{\text{RGR}}$  and nitrogenase activity were significantly ( $P < 0,05$ ) greater at high (64  $\text{mg} \cdot \text{l}^{-1}$ ) Ca concentrations than those recorded at low (16  $\text{mg} \cdot \text{l}^{-1}$ ) Ca concentrations.

TABLE 38 - The effects of culture pH and Fe source on the growth rate ( $\overline{\text{RGR}}^*$ :  $\text{g.g}^{-1}.\text{day}^{-1}$ ) and nitrogenase activity ( $\text{EPR}^*$ :  $\text{nM. (g.f.wt)}^{-1}.\text{hr}^{-1}$ ) of *A. filiculoides* grown at two levels of Ca. [C.A. = Citric acid]

pH	Iron source	16 $\text{mg.}\ell^{-1}$ Calcium		64 $\text{mg.}\ell^{-1}$ Calcium	
		$\overline{\text{RGR}}$	EPR	$\overline{\text{RGR}}$	EPR
5,0	$\text{Fe}^{+++}$	0,219 $\pm$ 0,050	2380 $\pm$ 149	0,239 $\pm$ 0,060	2595 $\pm$ 128
	$\text{Fe}^{+++}$ + C.A.	0,231 $\pm$ 0,036	2505 $\pm$ 163	0,250 $\pm$ 0,037	2712 $\pm$ 203
	$\text{Fe}^{++}$	0,218 $\pm$ 0,039	2364 $\pm$ 182	0,246 $\pm$ 0,045	2638 $\pm$ 157
	$\text{Fe}^{++}$ + C.A.	0,230 $\pm$ 0,047	2472 $\pm$ 174	0,254 $\pm$ 0,038	2771 $\pm$ 183
	Fe-Na-EDTA	0,280 $\pm$ 0,062	3015 $\pm$ 238	0,272 $\pm$ 0,053	2947 $\pm$ 187
7,0	$\text{Fe}^{+++}$	0,081 $\pm$ 0,021	892 $\pm$ 86	0,124 $\pm$ 0,038	1330 $\pm$ 117
	$\text{Fe}^{+++}$ + C.A.	0,137 $\pm$ 0,036	1508 $\pm$ 123	0,152 $\pm$ 0,031	1675 $\pm$ 125
	$\text{Fe}^{++}$	0,212 $\pm$ 0,049	2322 $\pm$ 164	0,240 $\pm$ 0,062	2603 $\pm$ 113
	$\text{Fe}^{++}$ + C.A.	0,264 $\pm$ 0,072	2885 $\pm$ 189	0,264 $\pm$ 0,050	2910 $\pm$ 217
	Fe-Na-EDTA	0,284 $\pm$ 0,069	3098 $\pm$ 240	0,291 $\pm$ 0,081	3121 $\pm$ 248
9,0	$\text{Fe}^{+++}$	0,053 $\pm$ 0,016	574 $\pm$ 59	0,069 $\pm$ 0,019	746 $\pm$ 86
	$\text{Fe}^{+++}$ + C.A.	0,074 $\pm$ 0,020	814 $\pm$ 88	0,086 $\pm$ 0,023	938 $\pm$ 83
	$\text{Fe}^{++}$	0,182 $\pm$ 0,042	1962 $\pm$ 175	0,195 $\pm$ 0,039	2104 $\pm$ 238
	$\text{Fe}^{++}$ + C.A.	0,193 $\pm$ 0,041	2080 $\pm$ 209	0,201 $\pm$ 0,046	2182 $\pm$ 216
	Fe-Na-EDTA	0,204 $\pm$ 0,050	2245 $\pm$ 224	0,216 $\pm$ 0,038	2315 $\pm$ 159

[\* = mean  $\pm$  two standard errors]

At all pH levels, the addition of Fe plus a chelating agent (citric acid or EDTA) stimulated  $\overline{\text{RGR}}$  and nitrogenase activity. Values of  $\overline{\text{RGR}}$  and nitrogenase activity in those cultures provided with ferrous ( $\text{Fe}^{++}$ ) ions were always greater than those recorded for cultures supplied with ferric ( $\text{Fe}^{+++}$ ) ions. At pH 5, both  $\text{Fe}^{+++}$  and  $\text{Fe}^{++}$  ions gave rise to similar values of  $\overline{\text{RGR}}$  and nitrogenase activity. However, at pH 9, the  $\overline{\text{RGR}}$  and nitrogenase activity of cultures provided with  $\text{Fe}^{++}$  ions were three times higher than those containing  $\text{Fe}^{+++}$  ions. The control cultures, provided with ferric-monosodium-EDTA (Fe-Na-EDTA) as the iron source, had consistently higher  $\overline{\text{RGR}}$  values and nitrogenase activity than the other cultures at all pH and Ca levels.

The nutrient content of *A. filiculoides* was also affected by both the Ca content of the external medium and the type of Fe source supplied (Table 39). Plants grown in media with a high Ca concentration had correspondingly higher Ca contents at all the pH levels and Fe sources tested. However, the levels of P and Fe within these plants were always lower than the plants grown at low Ca levels. At all pH levels, the N content of Fe<sup>+++</sup>-grown plants increased with increasing external Ca concentrations. Plant N content decreased with increasing external Ca concentrations in Fe<sup>++</sup>-grown plants. The N content of those plants provided with Fe-Na-EDTA and high external Ca concentrations decreased over the whole pH range tested.

At pH 5, the addition of citric acid as a chelating agent to cultures of Fe<sup>++</sup>- and Fe<sup>+++</sup>-grown plants produced a low plant Ca content at both high and low external Ca concentrations. However, at both pH 7 and pH 9, the addition of a chelator to Fe<sup>+++</sup>- and Fe<sup>+++</sup>-grown cultures produced higher plant Ca concentrations than those cultures lacking a chelator. At all pH levels, the addition of a chelating agent to Fe<sup>++</sup>- or Fe<sup>+++</sup>-grown cultures resulted in a higher plant P, Fe and N contents than those cultures lacking a chelator. The highest plant N content (5,12 %) was recorded in the control *A. filiculoides* plants provided with Fe-Na-EDTA as Fe source.

#### 6.4 Discussion

During this investigation, the response shown by *A. filiculoides* plants to alterations of their chemical environment was manifested as changes in both nitrogenase activity and  $\overline{\text{RGR}}$  values. Measurements of these processes provided a means of quantifying the response of *A. filiculoides* to a particular nutrient treatment, and were used to compare the suitability of different chemical environments for sustaining *A. filiculoides* populations. The two major aspects of this investigation, the nutrient requirements of *A. filiculoides* and the influence of the fern on its environment are discussed separately.

TABLE 39 - The effects of culture pH, Ca concentration and Fe source on the Ca, P, Fe and N content of *A. filiculoides* plants. (All values as % of dry weight)

pH	Fe source	16 mg.l <sup>-1</sup> Ca in medium				64 mg.l <sup>-1</sup> Ca in medium			
		Ca	P	Fe	N	Ca	P	Fe	N
5,0	Fe <sup>+++</sup>	0,513	0,109	0,354	4,64	0,546	0,162	0,112	4,82
	Fe <sup>+++</sup> + C.A.	0,482	0,207	0,178	4,74	0,501	0,175	0,140	4,91
	Fe <sup>++</sup>	0,507	0,220	0,244	4,90	0,523	0,174	0,143	4,96
	Fe <sup>++</sup> + C.A.	0,484	0,239	0,183	4,95	0,505	0,198	0,169	5,03
	Fe-Na-EDTA	0,469	0,263	0,194	5,12	0,493	0,224	0,168	5,05
7,0	Fe <sup>+++</sup>	0,381	0,175	0,125	1,64	0,419	0,127	0,083	2,32
	Fe <sup>+++</sup> + C.A.	0,399	0,192	0,134	1,78	0,438	0,138	0,097	2,51
	Fe <sup>++</sup>	0,454	0,186	0,197	3,83	0,465	0,149	0,174	3,20
	Fe <sup>++</sup> + C.A.	0,477	0,202	0,211	4,14	0,486	0,171	0,174	3,47
	Fe-Na-ED-A	0,482	0,256	0,215	4,72	0,507	0,211	0,172	4,61
9,0	Fe <sup>++</sup>	0,346	0,149	0,086	1,45	0,375	0,091	0,088	1,65
	Fe <sup>+++</sup> + C.A.	0,363	0,154	0,097	1,63	0,394	0,102	0,103	1,76
	Fe <sup>++</sup>	0,419	0,168	0,104	2,40	0,424	0,104	0,120	2,18
	Fe <sup>++</sup> + C.A.	0,432	0,180	0,121	2,61	0,450	0,109	0,123	2,22
	Fe-Na-EDTA	0,458	0,147	0,136	4,43	0,471	0,105	0,135	4,16

#### 6.4.1 The nutrient requirements of *A. filiculoides*

In this investigation, laboratory experiments demonstrated the ability of *A. filiculoides* to tolerate a wide range of chemical environments and established both the optimum and minimum levels of each nutrient required to maintain high levels of growth and nitrogenase activity. In order to facilitate comparisons between these experimentally determined values and the results of chemical analyses from the study area, these data are summarized in Table 40. In evaluating these data, it must be emphasized that the data referring to tolerance and optimum levels were derived from batch cultures whilst the threshold values were obtained from continuous cultures.

TABLE 40 - Summary table showing a comparison between the chemical environment tolerated by *A. filiculoides* in laboratory studies with the chemical environment of the study area. The optimal range and threshold levels of each parameter, based on laboratory studies, are also included for comparison. [The values are given in mg.ℓ<sup>-1</sup> (salinity, macronutrients) or µg.ℓ<sup>-1</sup> (micronutrients)]

Parameter	Concentration range of each parameter				
	Tolerance	Optimum	Threshold	Study area	
				Open water	Under mat
Salinity	30-2000	178-407	ND	152-235	196-279
P	0,4-26	0,8-13	0,12	0,02-0,06	0,09-0,15
Ca	4-20	12-16	12	9,6-12,0	11,2-14,0
Mg	5-40	16-28	14,5	14,5-17,3	15,8-21,0
K	0,8-32	4-16	1,2	1,9-3,5	2,3-4,7
Fe	0,08-8	0,5-4	0,12	0,02-0,06	0,11-0,15
Na	1,6-204	25,5-102	ND	6-10	8-14
S	0,8-52	6,5-52	ND	11-20	16-25
Cl	2,6-336	21-168	ND	14-24	18-30
B	4-256	16-128	12,0	0,8-2,1	1,1-3,6
Mo	0,3-19	0,6-4,8	1,5	0,5-0,6	0,8-1,2
Mn	3,8-248	15,5-248	10,0	30-50	40-85
Co	0,6-30	1,2-9,4	0,8	5-10	10-20
Cu	0,15-120	1,3-5,1	0,8	35-60	50-120
Zn	0,3-150	2,9-47	1,6	20-35	40-85

[ND = not determined]

The nitrogen-free culture media tested in this study varied considerably, both in their ionic strength and the variety of nutrient salts employed. High values of  $\overline{\text{RGR}}$  and nitrogenase activity were only obtained in media whose salt concentration ranged between 178 and 407 mg.ℓ<sup>-1</sup> (Table 40). Salt concentrations less than or greater than this range resulted in low  $\overline{\text{RGR}}$  and nitrogenase activity values. This finding is supported by the results of Le Van and Sobachkin (1963) for *A. pinnata*, indicating that salinity or osmotic effects are important growth regulators. The concentrations of total dissolved salts encountered at all riverine sites in

the study area fell within the optimum range shown in Table 40, indicating that *A. filiculoides* would not normally be stressed by salinity or osmotic effects.

In initial experiments, the medium designed by Gorham *et al.* (1964), combined with the micronutrient formulation of Johnson *et al.* (1966), gave consistently higher values of  $\overline{\text{RGR}}$  and nitrogenase activity than the other media initially tested. Later tests showed that  $\overline{\text{RGR}}$  values in this nutrient medium (though slightly lower), were comparable to the  $\overline{\text{RGR}}$  values obtained with the medium designed by Peters and Mayne (1974a). Further optimization of the ASM-1 medium of Gorham *et al.* (1964) and the micronutrient combination of Johnson *et al.* (1966) in batch culture indicated that despite the ability of *A. filiculoides* to tolerate a wide range of nutrient concentrations, high values of  $\overline{\text{RGR}}$  and nitrogenase activity are maintained only over a rather narrow optimum concentration range for most macronutrients (Table 40). Above or below this range,  $\overline{\text{RGR}}$  and nitrogenase activity decrease sharply. The exceptions to this trend, P and Fe, maintain high levels of  $\overline{\text{RGR}}$  and nitrogenase activity over a much wider concentration range (Table 40). The final medium obtained was similar in formulation to that used by Peters and Mayne (1974a) and gave  $\overline{\text{RGR}}$  and nitrogenase activity values which were superior to any other medium tested. These rates ( $\overline{\text{RGR}} = 0,330 \text{ g.g}^{-1} \cdot \text{day}^{-1}$ ;  $\text{EPR} = 3215 \text{ nmoles C}_2\text{H}_4 \cdot (\text{g.f.wt})^{-1} \cdot \text{hr}^{-1}$ ) are comparable to the highest rates obtained by Peters *et al.* (1980) with *A. filiculoides*. The final formulation, having a total dissolved salts concentration of  $245 \text{ mg.l}^{-1}$  and designated Azolla-I, was used for all further nutrient studies in the laboratory.

Continuous culture experiments demonstrated that nutrient supply rate (a function of flow rate) is important, but that a certain minimum nutrient concentration or threshold level is also necessary. This agrees with the observations of Subudhi and Watanabe (1979; 1981) who investigated the P requirements of *A. pinnata* in continuous culture. In the present study, the threshold levels of the macronutrients Ca, Mg, K and P for optimum growth of *A. filiculoides* were found to be 12,0, 14,5, 1,2 and 0,12  $\text{mg.l}^{-1}$  respectively. These values are lower than the levels of these elements found to be necessary for *A. pinnata* by Yatazawa *et al.* (1980). However, Yatazawa *et al.* (1980) employed a batch culture technique, which would account for their higher threshold levels. Subudhi and Watanabe (1981)

found that the P threshold concentration for *A. pinnata* in continuous culture was  $0,06 \text{ mg.l}^{-1}$ , half that of *A. filiculoides*. These authors also mentioned that a strain of *A. filiculoides* which they obtained from Hawaii had a greater P requirement than any of their *A. pinnata* strains. This difference once again highlights the physiological differences between different species of *Azolla* and supports the findings of this study.

For full development of nitrogenase activity, the threshold levels of Ca, Mg, K and P are 8,0, 9,7, 1,2 and  $0,12 \text{ mg.l}^{-1}$  respectively. With the exception of P and K these levels are slightly lower than the threshold levels for optimum growth. This is in contrast to the report of Yatazawa *et al.* (1980) who indicated that much higher concentrations of these elements were required for nitrogen fixation in *A. pinnata*. Again, this discrepancy is probably due to their use of batch culture techniques.

The flow rates employed in laboratory continuous cultures ( $0,5$  to  $4,0 \text{ l.hr}^{-1}$ ) were lower than even the lowest flow rates measured in the field during winter ( $7200 \text{ l.hr}^{-1}$ ). Indeed, high summer-flow rates, recorded during the annual floods, were up to 1,8 million times higher than the flow rates used in the laboratory. However, the effect of river flow on *A. filiculoides* plants depends not only on the flow rate but also the morphometry of the sampling site. The riverine sites monitored during this study varied in width from 2 m to 10 m (Table 1), and since the catchment areas of these sites were relatively small, high flow rates are usually of short duration (1-3 days). Low flow rates are recorded for the major portion of each year. *Azolla filiculoides* proliferates during the periods of low flow and the mats that develop become partly anchored to stands of marginal vegetation (predominantly *Phragmites* sp. and *Typha capensis*). During these periods of low river flow, the width of the river or stream causes the effective rate of flow beneath each unit area of *A. filiculoides* mat to approximate the flow rates used in laboratory continuous cultures. The results of these continuous culture experiments can therefore be related to that proportion of the year (> 90 %) when *A. filiculoides* populations in the field are subjected to low river flows.

In its natural environment, nutrient availability to *A. filiculoides* is regulated by the hydrochemistry of the water which in turn is dependent on

the hydrological cycle and the regional geology. Water samples from all of the riverine sites in the study area have a similar chemical composition, with some minor differences due to characteristics of the local geology and soils. All these streams and rivers carry a high load of suspended material, predominantly clay and silt, containing relatively large quantities of adsorbed cations. Seasonal differences in the hydrochemistry of these sites are due to seasonal changes in the hydrological cycle, with high summer flows being particularly important. The concentrations of the different cations and anions in the water from riverine sites are comparable to the values obtained for water samples from the Kraai and Caledon rivers by Keulder (1974). The ionic dominance order of the riverine water samples and the high proportions of total cations adsorbed onto clay and silt particles reported by Keulder (1974) in the Caledon and Kraai rivers and Stegmann (1974, 1975) for the water of the Hendrik Verwoerd Dam, were in agreement with the findings of this study. These adsorbed nutrients can, therefore, provide an important extra source of nutrients for *A. filiculoides* plants growing in the riverine sites. By contrast, the farm dam stations are not subjected to the same hydrological cycle as the riverine sites, and contain far lower levels of adsorbed nutrients. In these sites, dissolved nutrients form the largest proportion of the total nutrient supply.

At all riverine and farm dam sites, concentrations of micronutrients and P in the open water sections are always at very low levels (Table 40), often at the limit of analytical detection. However, water from beneath established mats of *A. filiculoides* has considerably higher micronutrient and P concentrations (Table 40). Seasonal differences in the concentrations of nutrients at the open water sites are generally low.

When the chemical analyses of the sampling stations are compared with the nutrient concentrations required to sustain optimal growth in the laboratory (Table 40), it was apparent that the concentrations of Ca and Mg are marginally sub-optimal though still high enough to sustain moderate  $\overline{\text{RGR}}$  values. The field concentrations of K, P and Fe are very low and could possibly be limiting *A. filiculoides* growth. However, the low K concentration at field sampling sites is greater than the threshold concentration measured in the laboratory (Table 40), and therefore its effect

on *A. filiculoides* would have been small. It is evident from the data presented in Table 40 that, for open-water sites, the concentrations of P and Fe are always well below the threshold level while Ca is slightly below threshold level only during summer. In addition, despite the higher nutrient concentrations found below an *Azolla* mat, the concentrations of Ca and P are below the threshold level only during summer while the Fe concentration is always at or just above the threshold level (Table 40). Therefore, based on the results of the laboratory experiments, the concentrations of Ca, P and Fe in the field are considered to be low enough to limit the growth of *A. filiculoides* whilst the field concentrations of Mg and K indicate an adequate supply of these macronutrients. With regard to the micronutrients, the data in Table 40 indicates that the concentrations of Mo and B in the field are below their respective threshold levels and therefore likely to be limiting *A. filiculoides* growth.

The results of the initial spiking experiments confirm that both P and Fe are the major limiting macronutrients in the field whilst Ca is also limiting the growth of *A. filiculoides*, but to a lesser extent. Additional spiking experiments also confirm that the micronutrients Mo and B are indeed limiting the growth of *A. filiculoides*. These results agree with the findings of other workers who have identified P and Fe as the macronutrients limiting the growth of *A. pinnata* in south-east Asia (Saubert, 1949; Le Van and Sobachkin, 1963; Moore, 1969; Watanabe *et al.*, 1977; Subudhi and Watanabe, 1979; Kulasooriya *et al.*, 1980) and *A. mexicana* and *A. filiculoides* in North America (Talley *et al.*, 1977; Talley and Rains, 1980).

In this study, adequate supplies of the macronutrients Ca, Mg, K and P were shown to be absolutely essential for optimum growth and nitrogenase activity in *A. filiculoides*. Deficiencies of these nutrients cause a greater reduction in nitrogenase activity than  $\overline{\text{RGR}}$ , thus supporting the observations of Le Van and Sobachkin (1963), Subudhi and Singh (1979a) and Yatazawa *et al.* (1980) on *A. pinnata* and Cohn and Renlund (1953) on *A. caroliniana*. Reported values for the minimum P concentration required to support optimal *Azolla* growth vary from 1,1 mg.l<sup>-1</sup> for *A. caroliniana* in Danish lakes (Olsen, 1972) to 2,5 mg.l<sup>-1</sup> (Yatazawa *et al.*, 1980) and 12,4 mg.l<sup>-1</sup> (Subudhi and Singh, 1979a) for *A. pinnata* in laboratory cultures. All of these values were determined with traditional batch

culture techniques and are considerably greater than the threshold level (0,12 mg.l<sup>-1</sup>) reported here for *A. filiculoides*.

In attempts to determine the effects of nutrient deficiencies on the  $\overline{\text{RGR}}$  and nitrogenase activity of different *Azolla* species, several workers (Bortels, 1940; Le Van and Sobachkin, 1963; Johnson *et al.*, 1966; Subudhi and Singh, 1979a; Yatazawa *et al.*, 1980) have experienced problems when using traditional batch culture techniques. In particular, the carry-over of nutrients during sub-culturing has led to a variety of interpretations as to the relative importance of individual nutrients. An additional feature, the ability of a plant to store nutrients excess to its requirements and then later<sub>A</sub><sup>rb</sup> transport and redistribute these nutrients within the plant, also contributes to the problem. Deficiencies of those nutrients which are relatively immobile within plants, such as Ca and Fe (Brouwer, 1965), are rapidly manifest in *A. filiculoides* both as foliar symptoms and dramatic decreases in both  $\overline{\text{RGR}}$  and nitrogenase activity. The appearance of deficiency symptoms due to a lack of other, more mobile, elements such as Mg, K and P (Brouwer, 1965), depends on the quantity of these nutrients already within the plant. The rapidity with which Ca and Fe deficiencies were manifested in this study indicate that *A. filiculoides* is more sensitive to sub-optimal concentrations of these nutrients.

In the present study, an adequate supply of Fe was found to be essential for growth and nitrogenase activity in *A. filiculoides*. In addition, it was found that the availability of dissolved Fe to *A. filiculoides* depends on both the concentration and ionic form in which the Fe is supplied, as well as the pH and calcium content of the culture medium. These results support the observations of Olsen (1972) who reported that *A. caroliniana* growing in small Danish lakes thrived where waters were anaerobic and Fe present in the reduced (ferrous) form. In those lakes which were not anaerobic and Fe present in the oxidized (ferric) form, the plants became chlorotic and died due to Fe deficiency. Yatazawa *et al.* (1980) demonstrated that symptoms of Fe deficiency in *A. pinnata* could be reversed with oxidized (ferric) Fe at an acidic pH but not at a neutral or alkaline pH. Olsen (1972) showed that at pH 4, ferric ions were so readily available to *Azolla* that Fe toxicity resulted, but this could be prevented by increasing the Ca concentration to balance the increased absorption of Fe. Olsen (1972) also reported that his results indicated competition between ferrous

and manganous ions in neutral to alkaline solutions and the absorption of both ions could be decreased by high Ca concentrations.

In the present study a long culture period was found to be necessary before *A. filiculoides*  $\overline{\text{RGR}}$  values and nitrogenase activity were significantly reduced as a result of micronutrient deficiencies. This feature can be attributed to the inherent mobility of micronutrients within plants (Brouwer, 1965). It was also found that deficiencies of Mn, Co and Mo specifically affect nitrogenase activity whilst a deficiency of B affects  $\overline{\text{RGR}}$ . In addition, a deficiency of either Mn, Cu or Zn caused only a slight (15 %) decrease in  $\overline{\text{RGR}}$  values and nitrogenase activity in *A. filiculoides*, presumably due to the unavoidable contamination of micronutrient elements from reagents and water under the present experimental conditions. These results support the reports of requirements shown by *A. filiculoides* (Bortels, 1940), *A. caroliniana* (Olsen, 1972) and *A. pinnata* (Le Van and Sobachkin, 1963; Yatazawa *et al.*, 1980) for Mo, *A. caroliniana* (Johnson *et al.*, 1966; Olsen, 1972) and *A. pinnata* (Yatazawa *et al.*, 1980) for Co, and *A. caroliniana* (Olsen, 1972) and *A. pinnata* (Yatazawa *et al.*, 1980) for B, Cu, Mn and Zn.

#### 6.4.2 The influence of *A. filiculoides* on the ecosystem

It is evident from the data summarized in Table 40 that nutrient concentrations in the water beneath a mat of *A. filiculoides* are always higher than the concentrations in the open water. This pointed to the possibility that mat development modifies the chemistry of the underlying water, increasing its nutrient content. Since mat development often results in most or all of the underlying waters becoming at least partly anaerobic, the most obvious source of nutrients is the decomposing lower layers of the mat and sedimented detritus. Similar increased nutrient concentrations beneath mats of floating hydrophytes have been reported by Mitchell (1970), Mitchell and Tur (1975), McVea and Boyd (1975) and Attionu (1976).

Experimental investigation of *in situ* decomposition rates confirm the rapid release of nutrients from both live and desiccated *A. filiculoides* plants. After an initial lag period, the decomposition of live *A. filiculoides* plants beneath an established mat of *A. filiculoides* occurs at a constant rate. The initial lag period of approximately five days noted

in this study may have been due to the use of initially fresh rather than truly senescent plant material. Since senescent plants are often attached to or held against the bottom of the *A. filiculoides* mat by their buoyant aerenchymatous structure, nutrients liberated during decomposition must be immediately available for uptake via the roots of upper, living *A. filiculoides* plants. The rates of nutrient release from the live *A. filiculoides* material obtained in this study are similar to those reported by Boyd (1970, 1971), Howard Williams and Junk (1976) and Rogers and Breen (1982) for other senescent aquatic macrophytes, despite the fact that this study was conducted in almost completely anaerobic waters ( $O_2$  concentration = 0,08 - 0,15 mg.l<sup>-1</sup>). The rates of decomposition of desiccated *A. filiculoides* material are also very similar to those obtained by Howard Williams and Junk (1976) and Rogers and Breen (1982). Particularly noticeable are the initial rapid loss rates of K, P and Fe. Thus, *A. filiculoides* plants which have been desiccated by the drying-up of a stream pool can rapidly liberate large quantities of K, P and Fe upon re-flooding. Since P and Fe are the primary limiting nutrients, this source of supply can be of great importance to the growth of *A. filiculoides*. An interesting feature of these studies on desiccated *A. filiculoides* material is the initial rapid increase in Ca and Mg content. Rogers and Breen (1982) also observed this phenomenon with decomposing *Potamogeton crispus* and attributed it to the adsorption of Ca and Mg ions onto the surface of the plant material by a cation exchange reaction. Thus, newly-flooded, desiccated *A. filiculoides* plants can adsorb Ca and Mg from the water, decreasing the availability of these nutrients. However, this effect would be temporary and the plant nutrients plus any adsorbed cations would be released during subsequent decomposition.

An important feature of mat formation in *A. filiculoides* is the effect that the established mat can exert on the underlying water by retarding the passage of atmospheric gases, with the consequent development of anaerobic or microaerophilic conditions. The presence of aerobic or anaerobic conditions in a water body has important consequences for the availability of nutrients, in particular Fe. In oxygenated waters of alkaline pH, almost all of the dissolved Fe content exists in the form of ferric ( $Fe^{+++}$ ) ions and the maximum solubility of  $Fe^{+++}$  ions in these waters is reported to be very low (0,02 to 0,03 mg.l<sup>-1</sup>; Olsen, 1972). Under oxygenated conditions, excess  $Fe^{+++}$  ions are precipitated out of solution to form

complex ferric-aluminium-phosphates in the sediments (Hutchinson, 1957). However, the apparent solubility of  $\text{Fe}^{+++}$  ions can be increased by the addition of an organic chelating agent and  $\text{Fe}^{+++}$  ions can also be readily adsorbed onto suspended silt and clay particles. The solubility of  $\text{Fe}^{+++}$  ions is also increased dramatically with decreased pH values (Olsen, 1972). The development of microaerophilic or anaerobic conditions, with a consequent shift in redox potential, provides conditions suitable for the transformation of  $\text{Fe}^{+++}$  ions to the reduced, ferrous ( $\text{Fe}^{++}$ ) form. This  $\text{Fe}^{++}$  form is up to 500 times more soluble in water than the oxidized,  $\text{Fe}^{+++}$  ion (Olsen, 1972), and this solubility is maintained over a wide pH range. If anaerobic water containing  $\text{Fe}^{++}$  ions is oxygenated, the unstable  $\text{Fe}^{++}$  ions slowly become oxidized to  $\text{Fe}^{+++}$  ions. These, because of their reduced solubility, rapidly precipitate out of solution. The stability of  $\text{Fe}^{++}$  ions in solution can also be increased by the addition of an organic chelating agent. The chelating agent combines with the  $\text{Fe}^{++}$  ions to form a solution containing complex Fe-organic molecules in which the  $\text{Fe}^{++}$  ions are still available for uptake by hydrophytes (Hutchinson, 1975; Sculthorpe, 1967). This solution of chelated  $\text{Fe}^{++}$  ions is relatively stable at low concentrations (ca.  $2.0 \text{ mg. l}^{-1}$ ; Hutchinson, 1957) in oxygenated waters.

A feature of the nutrition of *A. filiculoides*, the interactions between Ca and Fe, is also applicable to the situation which exists both in the open water and beneath mats of *A. filiculoides* at both riverine and farm dam sites in the study area. At the riverine site, the continual flow of water maintains low Ca and Fe concentrations. Here, the low Fe concentrations are often below the threshold level for optimum growth and therefore cause Fe limitation of *A. filiculoides* growth. High summer flow rates further decrease the Fe concentrations and therefore exacerbate this condition. During mat formation, the decomposition of senescent *A. filiculoides* plants results in increased concentrations of dissolved organic compounds. Since many of these compounds can function as chelating agents for Fe (Hutchinson, 1957; Sculthorpe, 1967), the quantity of dissolved Fe available for uptake by *A. filiculoides* is thus increased, whether the water is oxygenated or anaerobic. In these riverine sites, the inflow of oxygenated river water beneath the *A. filiculoides* mat mixes with lower anaerobic water. This results in a mixture of  $\text{Fe}^{+++}$  and  $\text{Fe}^{++}$  ions, both

dissolved and chelated, being available to *A. filiculoides*. However, the constant inflow (and consequent outflow) of water ensures that the Fe concentration remains low and does not continually rise. At the farm dam sites, increased Fe concentrations are counteracted by increased Ca concentrations which in turn prevent Fe accumulation and the development of toxicity symptoms. At the same time, this interaction between Ca and Fe also lowers the concentration of Fe available to *A. filiculoides* for growth, and results in Fe limitation. In the case of mat development on a farm dam, the anaerobic conditions ensure that all free or chelated Fe is in the  $\text{Fe}^{++}$  form. Because of evaporative concentration and continual decomposition, the concentration of  $\text{Fe}^{++}$  ions increases with time.

The combination of partly anaerobic (or microaerophilic) conditions with changed water pH values which develop beneath an *A. filiculoides* mat dramatically affect the supply of nutrients to *A. filiculoides* plants within the mat. Several other workers have recorded similar changes in pH and oxygen status beneath mats of *Eichhornia crassipes* (McVea and Boyd, 1975; Rai and Munshi, 1979), *Pistia stratiotes* (Okali and Attionu, 1974; Attionu, 1976) and *Salvinia molesta* (Mitchell, 1970; Mitchell and Tur, 1975; Bond and Roberts, 1978). In all of these cases, the establishment of a hydrophyte mat resulted in increased nutrient concentrations in the underlying waters. The formation of a multi-layered mat by *A. filiculoides* therefore represents a means by which the plant is able to modify the environment to its advantage, in particular by maintaining high rates of decomposition thus leading to increased rates of nutrient recycling. Without the formation of a mat and the subsequent increase in nutrient concentrations in the underlying waters, the concentrations of almost all nutrients would be far lower than the required threshold level. This would cause a decline in  $\overline{\text{RGR}}$  values and nitrogenase activity and the consequent death of the plants.

## CHAPTER 7

### THE COMPONENTS OF THE SYMBIOTIC ASSOCIATION

#### 7.1 INTRODUCTION

The principal features of symbiosis are a degree of intimate association and a condition of mutual benefit or dependence between the constituent organisms (Vincent, 1967). Indeed, the achievement and success of a symbiotic association depends on the degree to which the reciprocal requirements of each partner in the association are met. These demands range from simpler requirements in the case of loose associations to more specific demands in connection with the joint provision of specialized morphological or anatomical structures.

In order to assess the degree of interdependence shown by the component organisms of a symbiotic association, the individual organisms must be isolated and then recombined after their individual growth requirements have been established (Ashton and Walmsley, 1976). The close association between members of the genus *Azolla* and the endophytic Cyanobacterium *Anabaena azollae* has long been recognized as symbiotic (Peters, 1975). Here, despite the interest stimulated by the agronomic implications of the nitrogen-fixing endosymbiont, the symbiotic mechanisms involved in the association have not been completely elucidated (Moore, 1969). In particular, difficulty in obtaining the endosymbiont in axenic culture and affecting a successful recombination of the isolated alga with the alga-free fern have greatly hampered progress (Walmsley *et al.*, 1973; Peters, 1976).

This investigation was designed to:

- (a) isolate and grow the fern and algal components of the association in axenic culture,
- (b) determine the environmental and nutrient requirements of the individual organisms, and
- (c) recombine the isolated components to reform the symbiotic association.

## 7.2 LITERATURE REVIEW

The relationship between *Anabaena azollae* and members of the genus *Azolla* was originally referred to as a "space parasitism" (Sadebeck, 1902; Oes, 1913; Huneke, 1933). This outdated description has since been replaced by the more accurate term "symbiosis" (Lang, 1965; Moore, 1969), indicating a mutually beneficial association. Recent studies by Peters and Mayne (1974a, b), Duckett *et al.* (1975), Hill (1975, 1977), Peters (1975, 1976, 1977), Peters *et al.* (1978), Newton and Herman (1979), Tyagi, Ray, Mayne and Peters (1981) have contributed much to our knowledge of the association. These studies have provided evidence that both the fern and the alga benefit from the association.

Benefits conferred on the alga by the fern are a degree of physical (and perhaps chemical) protection, and the provision of energy substrate and all mineral nutrients except nitrogen (Moore, 1969; Peters and Mayne, 1974b; Peters, 1975, 1976, 1977). *Anabaena azollae* contains the photosynthetic pigments chlorophyll *a* and phycocyanin (Peters and Mayne, 1974a; Becking, 1976a). The alga contains 7 to 13 % of the total chlorophyll within the association (Peters and Mayne, 1974a) and phycocyanin, a water-soluble phycobilin pigment, can trap light of low intensity within the leaf cavity and transfer the trapped energy on to chlorophyll *a* (Ray, Mayne, Toia and Peters, 1979). Despite the significant proportion of the total chlorophyll *a* held within the symbiont, the alga may be unable photosynthetically <sup>to</sup> support its level of nitrogen fixation (Peters, 1977). If this assumption is true, the alga must be able to supplement its energy requirements by either mixotrophic activity or by transfer of carboxylated compounds from the fern.

There is limited experimental proof of heterotrophism in *A. azollae* (Newton and Herman, 1979), though such evidence is available for other endophytic blue-green algae, e.g. the endosymbionts from cycad root nodules (Winter, 1935; Douin, 1953; Hoare, Ingram, Thurston and Walkup, 1971; Fogg *et al.*, 1973), lichens (Hitch and Millbank, 1975) and free-living *Nostoc muscorum* (Allison, Hoover and Morris, 1937), all of which are members of the heterocystous family *Nostocaceae*. Inorganic culture media have also been successfully utilized for the isolation of nitrogen-fixing blue-green algae from symbiotic associations with cycads (Hoare

*et al.* 1971; Caiola, 1975), lichens (Hitch and Millbank, 1975), bryophytes (Rodgers and Stewart, 1977; Stewart, 1978) and angiosperms (Silvester and Smith, 1969). In the above reports, the isolated endosymbionts were grown on inorganic media lacking a source of combined nitrogen. These reports also indicated that the algal symbionts were capable of both autotrophic and heterotrophic growth.

Benefits conferred on the fern by the alga could include the provision of organic growth-promoting substances, as suggested by Schaede (1947) and Hill (1975), as well as the supply of fixed nitrogen (Peters and Mayne, 1974b). Provision of the total nitrogen requirement of the association by the alga must confer a marked competitive advantage to the fern in waters containing low levels of combined nitrogen.

Alga-free plants of *Azolla* have rarely been found in nature (Moore, 1969) though a few occurrences have been reported (Marsh, 1914; Huneke, 1933; Shen, 1960; Hill, 1977). Different degrees of success in freeing *Azolla* of *Anabaena* have been claimed for the following methods: low temperature (Huneke, 1933), very dilute culture medium (Huneke, 1933; Schaede, 1947), low light intensity (Schaede, 1947), change from low light intensity on nitrogen-free medium to high light intensity on media containing combined nitrogen (Hill, 1975), nitrogen deficiency and treatment with calcium hypochlorite (Huneke, 1933). Nickell (1958) developed a dependable method of producing alga-free *Azolla* through the use of three sequential antibiotic treatments, each of one week's duration. This method has been successfully used by Johnson *et al.* (1966), Walmsley *et al.* (1973), Peters and Mayne (1974a, b) and Peters (1975, 1976). Hill (1975, 1977) produced alga-free *Azolla* by transferring the plants from low (1250 Lux) to high (10 kLux) light intensity, a method very similar to that reported by Schaede (1947). Duckett *et al.* (1975) successfully produced alga-free *Azolla* plants by culturing small portions of excised stem apices which had been freed of alga by surface sterilization and repeated washings. Treatments with alcohol (Huneke, 1933) and ultraviolet irradiation (Flint, 1942) were unsuccessful.

Alga-free plants of *Azolla* are more compact in size and shape, grow more slowly than the intact association and require a source of combined nitrogen

(Hill, 1975; Peters, 1976). These features underline the importance of the alga for normal growth of the fern and suggest the existence of a growth-promoting substance or substances which are transferred from the alga via the apical meristem and/or the leaf cavities.

The isolation of experimental quantities of *A. azollae* from *Azolla* fronds has long proved to be a problem. Early methods using single leaf squashes (Venkataraman, 1962) and micromanipulation techniques were time-consuming and often ineffective. Recently, however, two techniques have been developed which provide large quantities of *A. azollae*. The first of these involves squashing *Azolla* fronds with a teflon roller ("the gentle roller method") followed by coarse filtering and centrifugation (Peters and Mayne, 1974a). The second method consists of prolonged enzymatic digestion of *Azolla* fronds with cellulase and pectinase, followed by several cycles of screening and vortexing until only algal packets surrounded by a filmy limiting envelope remain (Peters, 1976; Newton and Herman, 1979).

The difficulty in culturing isolated *A. azollae* has been a major obstacle in elucidating mechanisms of the symbiosis, and numerous authors have reported that the phycobiont cannot be grown apart from the host (Oes, 1913; Shields and Durrell, 1964; Lang, 1965; Walmsley *et al.*, 1973; Peters and Mayne, 1974a, b; Peters, 1975, 1976; Hill, 1975; Singh, 1977a). However, several claims of isolation and independent growth have been made (Canabeus, 1929; Vouk and Wellisch, 1931; Huneke, 1933; Bortels, 1940; Shen, 1960; Venkataraman, 1962; Weiringa, 1968; Becking, 1976b; Holst and Yopp, 1979b). Many of these isolates were made from non-sterile material and are suspect since the isolates may in fact have been epiphytic contaminants. Indeed, all claims of successful isolation and growth have been disputed by those workers who have been unable to culture isolated *A. azollae*.

Attempts to re-establish the symbiotic association, and thereby complete all of Koch's postulates, by infecting alga-free *Azolla* fronds with cultured *A. azollae* have been unsuccessful (Huneke, 1933; Bortels, 1940). Because of the possibility that the physiology of the cultured phycobiont may have been drastically altered during the culture process, recombination would be difficult to achieve (Peters, 1975).

### 7.3 MATERIALS AND METHODS

#### 7.3.1 Isolation and culture of *Anabaena azollae*

Three different methods of isolating *A. azollae* from *Azolla filiculoides* were tested during this study. All of the methods required the use of a nutrient medium to permit initial growth of the isolated alga. A N-free modification of the BG-11 medium of Stanier, Kunisawa, Mandel and Cohen-Bazire (1971) was chosen since this medium selected for nitrogen-fixing organisms. The composition of this medium is shown in Table 41. The pH of this medium was adjusted to 7,0 with either 1N HCl or 1N NaOH before sterilization. Solid media contained 1,5 % agar. Prior to use, all *Azolla* fronds were surface-sterilized with a 1 % (w/v) solution of NaOCl containing 0,1 % (v/v) teepol as a wetting agent for 15 minutes, followed by several rinsings in large volumes of sterile N-free BG-11 medium.

TABLE 41 - Composition of the modified nitrogen-free BG-11 medium of Stanier *et al.* (1971) used for the isolation and culture of *Anabaena azollae*

Stock solution*	Compound	Culture solution concentration (mg.l <sup>-1</sup> )
1	MgSO <sub>4</sub> ·7H <sub>2</sub> O	75
2	CaCl <sub>2</sub> ·2H <sub>2</sub> O	36
3	K <sub>2</sub> HPO <sub>4</sub>	40
4	Na <sub>2</sub> CO <sub>3</sub>	20
5	Fe-Na-EDTA	8
6	{ H <sub>3</sub> BO <sub>3</sub>	2,86
	{ MnCl <sub>2</sub> ·4H <sub>2</sub> O	1,81
	{ ZnSO <sub>4</sub> ·7H <sub>2</sub> O	0,222
	{ Na <sub>2</sub> MoO <sub>4</sub> ·2H <sub>2</sub> O	0,390
	{ CuSO <sub>4</sub> ·5H <sub>2</sub> O	0,079
	{ CoCl <sub>2</sub> ·6H <sub>2</sub> O	0,0494

[\*Concentrations were increased 100-fold and made up into stock solutions 1 to 6 as shown. All chemicals were of analytical grade]

(a) Leaf squash technique

Individual leaves were excised from a sterilized frond with a microscalpel, split longitudinally, and gently squashed, permitting the algae to protrude from the incision. Each treated leaf was then placed in a separate test-tube containing 5 ml of sterile N-free BG-11 medium. All the tubes were then placed in a constant environment room and incubated at 25 °C and low light intensity (500 Lux), and examined daily for signs of algal growth.

(b) Multiple centrifugation

Under sterile conditions, 4 to 5 g of *A. filiculoides* material were macerated in a blender for several minutes with 100 ml of sterile N-free BG-11 medium. The resulting slurry was then centrifuged at approximately 1000 X g in a laboratory centrifuge. The resulting pellet was two-layered, the lower layer consisting of *Azolla* fragments while the upper layer contained mostly algal filaments. The supernatant liquid was decanted and a portion of the upper pellet layer resuspended in sterile N-free BG-11 medium. This suspension was then centrifuged at approximately 500 X g. Again, the resulting pellet consisted of two layers, the upper layer being virtually pure *A. azollae* filaments. A portion of this upper layer was resuspended in sterile N-free BG-11 medium and single drops of this suspension were dispensed to test-tubes containing 5 ml of sterile N-free BG-11 medium. The test-tubes were incubated and examined daily for signs of algal growth as described above.

(c) Micromanipulation

Whole algal colonies were surgically removed from *Azolla* leaf cavities with sterile glass micropipettes under a 20 X Wild dissecting microscope. Each colony was inoculated into a separate test-tube containing 5 ml sterile N-free BG-11 medium. The tubes were incubated and examined for algal growth as described above.

After a period of three weeks, those tubes showing signs of algal growth were subjected to a temperature of 47 °C (+ 1 °C) for 100 minutes to kill contaminating bacteria (Weiringa, 1968). Each tube was then cooled to room temperature, shaken to disperse the algal filaments and two drops of each

algal suspension inoculated onto N-free BG-11 agar plates. Algal cells from these cultures were repeatedly streaked to eliminate any residual adhering bacteria.

Culture purity was checked by inoculating yeast extract + glucose agar with drops of algal suspension, followed by incubation at 25 °C. These plates were then examined daily for 10 days for bacterial colonies. The absence of bacterial colonies on the agar plates was taken to indicate the absence of viable contaminating bacteria in the algal isolates. As a further check of culture purity, portions of the algal isolates were also examined microscopically under phase-contrast illumination for the presence of contaminating bacteria.

### 7.3.2 Isolation and culture of the fern component

Four different methods of isolating the fern component of the symbiotic association (i.e. removing the algal component) were tested during this study. All the methods required a medium containing a source of combined nitrogen to permit growth of the alga-free fern. The Azolla-I medium (described in Section 6.3.3), modified by the addition of 10 mg.l<sup>-1</sup> NO<sub>3</sub>-N as NaNO<sub>3</sub>, was used for this purpose. This medium was designated Azolla-I(+N). Prior to use, all *A. filiculoides* material was surface-sterilized as described in Section 7.3.1.

#### (a) Environmental modification

Five experiments, employing different variations of temperature and light intensity (listed below), were carried out to determine whether sudden changes in growth conditions could produce alga-free *Azolla* plants as described by Huneke (1933) and Schaede (1947). Plants of *A. filiculoides* were acclimatized for two weeks on Azolla-I(+N) medium at the first level of light intensity and temperature. After this acclimatization period, the levels of light intensity and temperature were doubled (i.e. to the second level) and the plants were then grown for a further two weeks. Portions of plants were examined under a dissecting microscope for the presence or absence of algal colonies. After the second two week period, the temperature and light intensity were suddenly dropped to the original levels and

the plants were again examined microscopically after a few days. The different levels of light intensity and temperature tested in the five experiments were: 5 kLux/10 °C → 10 kLux/20 °C → 5 kLux/10 °C, 10 kLux/10 °C → 20 kLux/20 °C → 10 kLux/10 °C, 15 kLux/10 °C → 30 kLux/20 °C → 15 kLux/10 °C, 20 kLux/10 °C → 40 kLux/20 °C → 20 kLux/10 °C, 30 kLux/10 °C → 60 kLux/20 °C → 30 kLux/10 °C.

(b) Apical meristem culture

Apical portions (approximately 2 mm long) of surface-sterilized *A. filiculoides* fronds were surgically excised, rinsed vigorously in several changes of sterile distilled water and transferred to test-tubes containing 5 ml of sterilized Azolla-I(+N) medium. Each tube was examined daily for signs of growth. Those apices which survived the treatment and grew were examined microscopically for the presence or absence of algal colonies. This process was also repeated with 2 mm apices from which all the leaves (but not leaf primordia) had been removed. All incubations were carried out at 25 °C and at a light intensity of 5 kLux.

(c) Megaspore culture

Mature megaspores with attached massulae, collected both in the field and from laboratory cultures, were surface-sterilized with NaOCl, using the procedure described above for *Azolla* fronds (Section 7.3.1). The indusium cap of each megaspore was then gently excised with a micro-scalpel and the cluster of algal akinetes at the apex of the pseudocellular float structures were carefully scraped away. Adhering algal cells were removed by gentle rinsing in sterile distilled water and each megaspore was transferred to a test-tube containing 5 ml of sterile Azolla-I(+N) medium. The tubes were examined daily for signs of megaspore germination.

(d) Antibiotic treatment

A modification of the antibiotic treatment used by Nickell (1958) was employed to free *A. filiculoides* of the endophytic alga. Surface-sterilized fronds were grown on Azolla-I(+N) medium and subjected to three consecutive antibiotic treatments, each of one week's duration.

During each week-long treatment, the basal medium was supplemented with a single antibiotic. At the end of each week, the fronds were rinsed with sterile medium and transferred to the next treatment. The sequence and concentration of antibiotic treatments used was (a)  $25 \text{ mg.l}^{-1}$  Na penicillin G, (b)  $4 \text{ mg.l}^{-1}$  terramycin, and (c)  $5 \text{ mg.l}^{-1}$  streptomycin sulphate. After this sequence of treatments, each frond was rinsed with sterile medium and transferred to a 150 ml capacity Erlenmeyer flask containing 75 ml of sterile Azolla-I(+N) medium. All the flasks were incubated at  $25^{\circ}\text{C}$  and 5 kLux, and the medium was replaced by siphoning at three-day intervals to avoid disturbing the plants.

Alga-free fronds of *A. filiculoides* were maintained in 500 ml capacity, cotton wool-capped Erlenmeyer flasks containing 200 ml of sterile Azolla-I(+N) medium. Again, the medium was replaced by siphoning every three days to avoid disturbance to the plants. Portions of fronds were examined microscopically under phase-contrast illumination for the presence of contaminating algae and bacteria.

### 7.3.3 Determination of growth requirements for the isolated fern and algal components

Several series of experiments were carried out to investigate the range of environmental and nutrient conditions tolerated by the isolated fern and algal components as well as the requirements for optimal growth. Aseptic cultures of the isolated fern and algal components were grown in large phytotrons (described in Section 2.5.2.1) and subjected to a wide range of photoperiods, temperatures and light intensities (described below). Nutrient requirements were ascertained by comparing rates of growth on different concentrations of a range of media. In these experiments, the algae were grown on nitrogen-free media while the fern component was grown on media containing a source of combined nitrogen. Variations in atmospheric conditions within the culture flasks could be achieved by altering the ratio of the component gases ( $\text{N}_2$ ,  $\text{O}_2$ ,  $\text{CO}_2$ ) with a Matheson model 665 gas proportioner. These gases were of the highest purity available and were obtained from African Oxygen Ltd, Port Elizabeth. The ranges of each environmental variable tested were: photoperiod: OL/24D to 24L/OD; constant temperature:  $5^{\circ}\text{C}$  to  $35^{\circ}\text{C}$ ; different day

and night (D/N) temperatures (keeping the thermoperiod the same as the photoperiod): 10/5 °C to 40/30 °C; light intensity: 1 kLux to 60 kLux; pH: 3 to 10. In each experiment, one variable was tested at several different levels while the other variables were held constant. In the case of photoperiod, several experiments were conducted at a particular photoperiod and then repeated when the photoperiod was changed at the conclusion of each trial.\*

#### 7.3.4 Recombination of the isolated fern and algal components

Five methods of recombining the isolated fern and algal components were tested in attempts to reconstitute the symbiotic association.

##### (a) Liquid association

Alga-free plants of *A. filiculoides* and pure cultures of *Anabaena azollae* were grown together in both N-free Azolla-I medium and Azolla I medium supplemented with  $2 \text{ mg} \cdot \text{l}^{-1} \text{ NO}_3\text{-N}$  as  $\text{NaNO}_3$ . The mixed cultures were maintained in 1 l capacity Erlenmeyer flasks containing 500 ml of sterile liquid medium. The flasks were placed on a Bühler rotary shaker (orbit radius 3 cm) and shaken at 15 revolutions per minute to prevent sedimentation of the algal filaments. The fern fronds were examined at two day intervals for signs of reinfection and growth.

##### (b) Micromanipulation

The apical meristem regions of alga-free plants were injected with small (5  $\mu\text{l}$ ) quantities of a dense suspension of *A. azollae* filaments. In a second series, 2  $\mu\text{l}$  of a dense *A. azollae* suspension were injected between each of the upper and lower leaf lobes nearest the apical meristem. Treated plants were transferred aseptically to N-free Azolla-I media and checked for signs of growth.

##### (c) Surface spray

Alga-free plants growing on Azolla-I(+N) medium were sprayed once a day for 10 days with a dense suspension of *A. azollae* filaments. Every second day, samples of the treated fronds were transferred to N-free Azolla-I medium and checked for signs of growth.

(d) Natural association

Alga-free plants and symbiotic *A. filiculoides* plants were grown together on Azolla-I medium supplemented with  $2 \text{ mg} \cdot \text{l}^{-1} \text{ NO}_3\text{-N}$  as  $\text{NaNO}_3$ . The addition of the source of combined nitrogen prevented the death of alga-free plants. Again, samples of the original alga-free plants were removed every two days and examined for signs of algal reinfection.

(e) Megaspore infection

Mature megaspores of *A. filiculoides* were surface-sterilized and the cluster of *A. azollae* akinetes removed as described in Section 7.3.2(c). Each megaspore was then gently rinsed in sterile distilled water and transferred to a test-tube containing 5 ml of a dense culture of *A. azollae*. The tubes were incubated at 5 kLux and  $20^\circ \text{C}$  for three weeks and the megaspores were examined every two days for signs of reinfection and germination.

## 7.4 RESULTS

### 7.4.1 Isolation of the fern component

All attempts to obtain alga-free *Azolla* plants by modifications of the external temperature, light intensity and nutrient concentration were unsuccessful. In a few cases (high temperature and high light intensity) the *A. filiculoides* plants died. Otherwise, the plants remained green and healthy though rates of growth and nitrogenase activity were reduced. Microscopic examination revealed that all these plants contained apparently healthy *A. azollae* colonies within their leaf cavities.

A few excised apical meristems grew for up to 8 days but then became chlorotic and died. All those apices where the leaves had been excised died within 3 days. None of the megaspores which had been freed of algal cells survived the treatment.

The use of antibiotic treatments was the most successful method and gave rise to alga-free plants of *A. filiculoides*. These plants did not reduce acetylene and required a source of combined nitrogen for growth since without this they died within 10 days on N-free media. The alga-free

fronds were much more compact than those which contained the symbiotic alga (Plate 21a) and grew more slowly. During an 18 month period, none of these plants regained their ability to reduce acetylene. Microscopic examination of the leaf cavities revealed them to be free of algal cells, but showed that bacteria and the secretory hair cells were still present (Plate 21b).

#### 7.4.2 Isolation and characterization of *Anabaena azollae*

Initial attempts to isolate *A. azollae* by means of leaf squashes or maceration and repeated centrifugation were uniformly negative. Extensive bacterial growth occurred in all liquid cultures and the algal cells appeared shrunken and discoloured under the microscope. When the process was repeated with agar plates containing both simple and complex media, only bacterial colonies developed. Liquid and solid media incorporating *Azolla* extracts and a variety of gas atmospheres containing different concentrations of CO<sub>2</sub> and O<sub>2</sub> were also unsuccessful in stimulating algal growth.

Successful isolation of *A. azollae* was only achieved with the micro-manipulation method. Here, only 8 of the original 275 inocula showed signs of algal growth after 10 days. The algal cells in these tubes appeared pale brownish at first, but after 18 days turned pale green and were easily visible to the naked eye. Two of these isolates did not survive the heat treatment designed to eliminate bacterial contamination. The six surviving isolates, designated as strains RU/PA 1 to 6, were freed of residual adhering bacteria by repeated streaking on agar plates and grew well on both solid and liquid N-free media. Microscopic examination and enrichment tests indicated that all six strains were free of contaminating bacteria.

Morphologically, the six strains of *A. azollae* were identical both in cell size and heterocyst frequency. On solid media, each of the six isolates had a similar growth form, consisting of characteristically whorled and twisted bundles of filaments (Plate 21c). In liquid media, the algal colonies tended to aggregate and precipitate, and then adhered to and grew along the sides and base of the culture vessel.

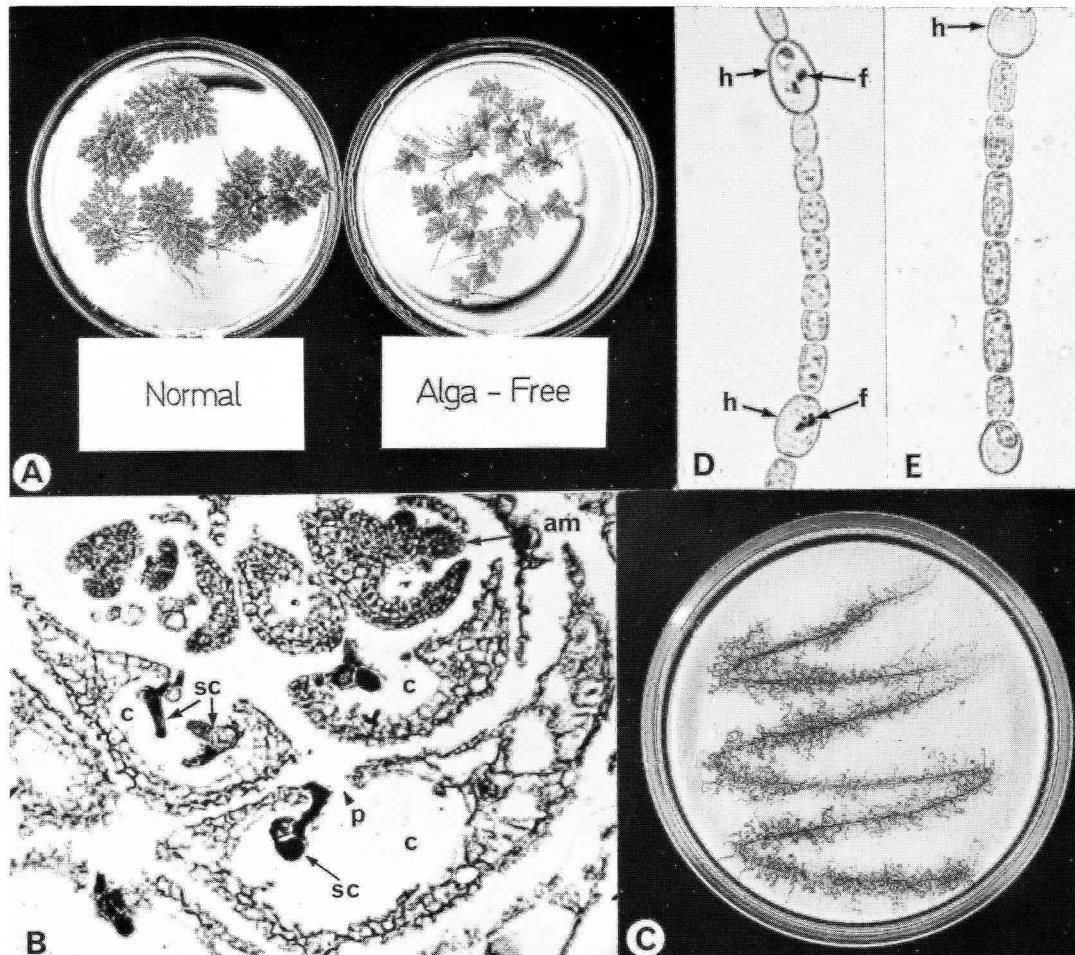


PLATE 21 : The isolated components of the symbiotic association.

A: Comparison of symbiotic and alga-free plants of *A. filiculoides*. After six months growth on Azolla-I(+N) medium, alga-free plants are small, compact, brittle and chlorotic while the symbiotic plants are larger, darker and freely-branching. B: (X50) LS of alga-free branch apex showing apical meristem (am) and empty leaf cavities (c) with secretory cells (sc) and pore (p). C: Typical growth form of *A. azollae*, five days after inoculation onto solid media, with aggregated whorls of filaments clearly visible. D: (X800) Filament of *A. azollae* after 20 minute treatment with triphenyl tetrazolium chloride (TTC), showing two intercalary heterocysts (h) containing conspicuous formazan crystals (f). E: (X600) Filament of *A. azollae*, three months after isolation, showing rounded heterocysts (h) and elongated vegetative cells.

All six isolates displayed similar changes in cell size and heterocyst frequency during the first three months of culture. Individual cells of each isolate enlarged, particularly in length, and heterocyst frequencies fell (Table 42). However, despite these changes, the heterocyst frequencies of the six *A. azollae* isolates were still 6 to 8 % higher than that of free-living *Anabaena flos-aquae* (Lyngb.) Bréb., obtained from the algal culture collection at the National Institute for Water Research, C.S.I.R., Pretoria (Culture Collection No. WR 14).

TABLE 42 - Morphological changes in isolated *Anabaena azollae*\* during the first three months of culture on nitrogen-free BG-11 medium

Time from isolation (days)	Vegetative cells		Heterocyst frequency (%)
	length (µm)	width (µm)	
0	7,1	5,5	28,3
10	8,7	5,8	25,1
30	10,8	6,0	20,6
60	12,0	6,0	18,2
90	12,5	6,1	17,9

[\*Data presented here for strain RU/PA 3; each value is a mean of twenty measurements]

#### 7.4.3 Growth requirements of the isolated components

Because of the problems in obtaining sufficient *A. azollae* material, all investigations of the alga's growth requirements could only be carried out six to nine weeks after the isolation of the alga. A good correlation was found to exist between high algal growth rate and high nitrogenase activity ( $r^2 = 0,92$ ;  $n = 10$ ). Therefore, to facilitate manipulation of the material, nitrogenase activity was used as a measure of the alga's growth rate. The use of nitrogenase activity as an indirect measure of the growth rate of free-living nitrogen-fixing blue-green algae has been successfully used by Stewart (1974).

Where possible, all plant and algal material was acclimatized for at least 20 days prior to each experiment (see Section 7.3.3 for details of the

treatments used). When this was impractical (e.g. severe conditions causing death), fern growth rates and algal nitrogenase activity were measured four days after initiation of the treatment. In most cases, only four replicates of each treatment were used due to the scarcity of material. Algal nitrogenase activity was expressed on the basis of total algal chlorophyll *a* content. Chlorophyll *a* content was determined, after extraction with 90 % ethanol, exactly as advocated by Wintermans and DeMots (1965).

With increasing photoperiod up to 16 hours, fern  $\overline{\text{RGR}}$  values and algal nitrogenase activity increased (Table 43). At longer photoperiods, the fern's  $\overline{\text{RGR}}$  continued to increase slowly while the alga's nitrogenase activity remained almost constant. In continuous darkness, the fern regressed rapidly and the algal nitrogenase activity dropped to zero after 24 hours.

TABLE 43 - The effect of increasing photoperiod on the  $\overline{\text{RGR}}$  of the alga-free fern and the nitrogenase activity of *A. azollae*. (Light intensity: 5 kLux; Temperature:  $20 \pm 1$  °C). [Each value is a mean of four replicates]

Photoperiod (hours)	$\overline{\text{RGR}}$ of alga-free fern ( $\text{g.g}^{-1}.\text{day}^{-1}$ )	Nitrogenase activity of <i>A. azollae</i> ( $\text{nmoles. (mg.chlorophyll)}^{-1}.\text{min}^{-1}$ )
0	0	0
2	0,009	15,2
4	0,015	26,7
8	0,029	54,0
12	0,055	71,3
16	0,072	80,1
20	0,080	84,9
24	0,085	87,8

When grown under conditions of constant temperature, both the alga-free fern and *A. azollae* showed maximum  $\overline{\text{RGR}}$  values (or nitrogenase activity) over a rather narrow temperature range (Table 44). The optimum temperature for growth of the alga-free fern was approximately 20 °C, while the optimum temperature "range" for *A. azollae* was between 20 and 25 °C. An interesting

feature of the degree of temperature tolerance shown by the two organisms was that *A. azollae* could tolerate a wider range of temperatures than the alga-free fern. Temperatures above 20 °C and below 10 °C caused a rapid decrease in the fern's  $\overline{\text{RGR}}$ , with zero growth recorded at 5 °C and 30 °C. In contrast, *A. azollae* still demonstrated measurable nitrogenase activity at 5 °C and 30 °C, but died at 35 °C.

TABLE 44 - The effect of different temperatures on the  $\overline{\text{RGR}}$  of the alga-free fern and the nitrogenase activity of *A. azollae*. (Photoperiod: 16L/8D; Light intensity: 5 kLux). [Each value is a mean of four replicates]

Temperature (°C)	$\overline{\text{RGR}}$ of alga-free fern (g.g <sup>-1</sup> .day <sup>-1</sup> )	Nitrogenase activity of <i>A. azollae</i> (nmoles.(mg.chlorophyll) <sup>-1</sup> .min <sup>-1</sup> )
5	0	5,6
10	0,014	34,4
15	0,059	57,9
20	0,076	81,8
25	0,034	82,2
30	0	20,7
35	0	0

When *A. azollae* and the alga-free fern were grown under a fluctuating day and night temperature regime (Table 45), the thermoperiod was set to 16L/8D to correspond with the photoperiod used. Here, both organisms were able to tolerate slightly higher maximum (day) temperatures (Table 45). Maximum growth (0,079 g.g<sup>-1</sup>.day<sup>-1</sup>) of the alga-free fern was recorded at a day/night temperature range of 25/15 °C, and zero growth was recorded at 10/5 °C and 35/25 °C. The highest *A. azollae* nitrogenase activity (87,3 nmoles C<sub>2</sub>H<sub>4</sub> produced (mg chlorophyll)<sup>-1</sup>.min<sup>-1</sup>) was measured at a day/night temperature of 30/20 °C. Above this level, and below 15/5 °C, nitrogenase activity declined dramatically. However, low nitrogenase activity values (2,1 nmoles C<sub>2</sub>H<sub>4</sub> produced (mg chlorophyll)<sup>-1</sup>.min<sup>-1</sup>) were still recorded at 40/30 °C.

Both the  $\overline{\text{RGR}}$  of the alga-free fern and the nitrogenase activity of *A. azollae* were suppressed by high light intensities (Table 46). With

increasing light intensity, the fern's  $\overline{\text{RGR}}$  increased to a maximum at around 10 kLux, and then declined to zero at 60 kLux. Algal nitrogenase activity was relatively high at the lowest light intensity used and increased to a maximum at 5 kLux. With higher light intensities, the algal nitrogenase activity declined to zero at 30 kLux.

TABLE 45 - The effects of different day and night temperatures on the RGR of the alga-free fern and the nitrogenase activity of *A. azollae*. (Photoperiod and thermo-period: 16L/8D; Light intensity: 5 kLux). [Each value is a mean of four replicates]

Day/night temperatures (°C)	$\overline{\text{RGR}}$ of alga-free fern ( $\text{g.g}^{-1}.\text{day}^{-1}$ )	Nitrogenase activity of <i>A. azollae</i> ( $\text{nmoles. (mg.chlorophyll)}^{-1}.\text{min}^{-1}$ )
10/5	0	23,3
15/5	0,011	47,2
20/10	0,048	69,1
25/15	0,079	80,5
30/20	0,032	87,3
35/25	0	25,2
40/30	0	2,1

TABLE 46 - The effect of increasing light intensity on the  $\overline{\text{RGR}}$  of the alga-free fern and the nitrogenase activity of *A. azollae*. (Photoperiod: 16L/8D; Temperature: 20 °C). [Each value is a mean of four replicates]

Light intensity (kLux)	$\overline{\text{RGR}}$ of alga-free fern ( $\text{g.g}^{-1}.\text{day}^{-1}$ )	Nitrogenase activity of <i>A. azollae</i> ( $\text{nmoles. (mg.chlorophyll)}^{-1}.\text{min}^{-1}$ )
1	0,013	23,9
2	0,039	67,8
5	0,070	79,8
10	0,072	39,9
15	0,061	11,2
30	0,030	0
45	0,006	0
60	0	0

Light quality markedly affected both the fern's  $\overline{\text{RGR}}$  and the alga's nitrogenase activity (Table 47). The  $\overline{\text{RGR}}$  values and nitrogenase activity in greenlight, though only 25 % and 42 % respectively of the control (white light), were considerably higher than the corresponding values for both red and blue light. Blue light alone was clearly unsuitable for both the fern and *A. azollae* and resulted in the death of the fern after six days.

TABLE 47 - The effect of light quality on the  $\overline{\text{RGR}}$  of the alga-free fern and the nitrogenase activity of *A. azollae*. (Photo-period: 16L/8D; Light intensity: 5 kLux; Temperature: 20 °C). [Each value is a mean of four replicates]

Filter transmission maxima (nm)	$\overline{\text{RGR}}$ of alga-free fern ( $\text{g} \cdot \text{g}^{-1} \cdot \text{day}^{-1}$ )	Nitrogenase activity of <i>A. azollae</i> ( $\text{nmoles} \cdot (\text{mg} \cdot \text{chlorophyll})^{-1} \cdot \text{min}^{-1}$ )
White (400-700)	0,074	81,4
Red (655)	0,004	2,9
Green (530)	0,019	34,5
Blue (450)	0,001*	1,0

[\* measured over first four days]

Algal nitrogenase activity was highest within the pH range 7,0-8,0 (Table 48). At pH 6,0 the activity was 51 % of the maximum and decreased further with decreasing pH until at pH 4,0 it was only 5 % of the maximum. Above pH 8,0 algal nitrogenase activity declined dramatically. When grown at pH 3,0 and 10,0 the algal cultures died within three days. Maximum  $\overline{\text{RGR}}$  values of the alga-free fern were recorded within the pH range 6,0-7,0.  $\overline{\text{RGR}}$  values at pH 5,0 and pH 8,0 were 84 % and 69 % respectively of the maximum. Outside of this pH range, the fern's  $\overline{\text{RGR}}$  dropped rapidly. At both pH 3,0 and 10,0 the alga-free fern died within four days.

Both the alga-free fern and *A. azollae* were highly sensitive to changes in the nutrient concentration of the external medium (Table 49). The highest fern  $\overline{\text{RGR}}$  values and algal nitrogenase activity were measured in full-strength (i.e. normal) medium, though half-strength media decreased  $\overline{\text{RGR}}$  values and nitrogenase activity below the rates in normal medium by

only 7 % and 4 % respectively. The alga-free fern  $\overline{\text{RGR}}$  values and algal nitrogenase activity were extremely low at both high (4 X) and low (1/8 X) nutrient concentrations. At four times the normal medium concentration, both the alga-free fern and *A. azollae* died within seven days.

TABLE 48 - The effect of different culture pH on the  $\overline{\text{RGR}}$  of the alga-free fern and the nitrogenase activity of *A. azollae*. (Photoperiod: 16L/8D; Light intensity: 5 kLux; Temperature: 20 °C). [Each value is a mean of four replicates]

Culture pH	$\overline{\text{RGR}}$ of alga-free fern (g.g <sup>-1</sup> .day <sup>-1</sup> )	Nitrogenase activity of <i>A. azollae</i> (nmoles.(mg.chlorophyll) <sup>-1</sup> .min <sup>-1</sup> )
3	0,001*	0,1**
4	0,021	3,8
5	0,068	19,2
6	0,079	41,6
7	0,081	79,3
8	0,056	81,0
9	0,021	24,5
10	0,001*	0,1**

[\*measured over first two days; \*\* measured on second day]

TABLE 49 - The effects of nutrient concentration (i.e. medium strength) on the  $\overline{\text{RGR}}$  of the alga-free fern and the nitrogenase activity of *A. azollae*. (Photoperiod: 16L/8D; Light intensity: 5 kLux; Temperature: 20 °C; pH: 7,0). [Each value is a mean of four replicates]

Medium strength	$\overline{\text{RGR}}$ of alga-free fern (g.g <sup>-1</sup> .day <sup>-1</sup> )	Nitrogenase activity of <i>A. azollae</i> (nmoles.(mg.chlorophyll) <sup>-1</sup> .min <sup>-1</sup> )
1/8 X	0,013	8,6
1/4 X	0,044	50,5
1/2 X	0,081	88,9
1 X	0,087	92,6
2 X	0,053	36,1
4 X	0,008*	2,4**

[\*measured over first four days; \*\* measured on fourth day]

The alga-free fern and *A. azollae* responded differently to changes in atmospheric oxygen concentration (Table 50). In this experiment the two levels of oxygen concentration chosen (1 % and 20 %) were selected so as to represent microaerophilic (1 % O<sub>2</sub>) and aerobic (20 % O<sub>2</sub>) conditions. These O<sub>2</sub> concentrations were achieved by altering the proportions of the three atmospheric gases used (N<sub>2</sub>, O<sub>2</sub>, CO<sub>2</sub>) with a Matheson model 665 gas proportioner. The highest fern  $\overline{\text{RGR}}$  values were recorded at an O<sub>2</sub> concentration of 20 %. At higher O<sub>2</sub> levels, the fern's  $\overline{\text{RGR}}$  remained constant. However, with decreasing O<sub>2</sub> concentrations there was a decrease in the fern's  $\overline{\text{RGR}}$  which registered 22 % of the maximum at an O<sub>2</sub> level of 1 %. The highest algal nitrogenase activity was measured at the lowest O<sub>2</sub> level used (1 %). Nitrogenase activity decreased as the O<sub>2</sub> level increased, stabilizing at O<sub>2</sub> levels close to normal atmospheric concentrations. However, an O<sub>2</sub> level of 30 % caused a further reduction (70 % of maximum value) in nitrogenase activity.

TABLE 50 - The effects of atmospheric oxygen content on the  $\overline{\text{RGR}}$  of the alga-free fern and the nitrogenase activity of *A. azollae*. (Photoperiod: 16L/8D; Light intensity: 5 kLux; Temperature: 20 °C; pH: 7,0). [Each value is a mean of four replicates]

Oxygen concentration (%)	$\overline{\text{RGR}}$ of alga-free fern (g.g <sup>-1</sup> .day <sup>-1</sup> )	Nitrogenase activity of <i>A. azollae</i> (nmoles.(mg.chlorophyll) <sup>-1</sup> .min <sup>-1</sup> )
1	0,018	105,7
2	0,035	93,4
5	0,049	87,8
10	0,069	84,1
15	0,078	80,3
20	0,083	78,6
25	0,080	80,2
30	0,081	70,9

The alga-free fern was found to be incapable of heterotrophic growth in the dark. On each attempt, when an exogenous carbon source (fructose, sucrose or glucose) was supplied, the cultures were eventually over-run by bacterial contamination and the alga-free fern material died.

Isolated *A. azollae* was capable of heterotrophic growth in the dark for a period of up to four days after which growth declined. In the dark, the alga demonstrated marked responses to different carbon sources (Table 51). Under both aerobic ( $O_2 = 20\%$ ) and microaerophilic ( $O_2 = 1\%$ ) conditions, additions of glucose or sucrose had equally inhibitory effects when compared to autotrophic control cultures. In contrast, the addition of fructose increased aerobic algal nitrogenase activity by 23% and microaerophilic rates by 85% relative to the control cultures. The rates shown in Table 51 were measured on the second day after initiation of cultures. The fructose-grown algal nitrogenase activity dropped to approximately the same as the control values after six days. Cultures grown on glucose and sucrose died on day five.

TABLE 51 - The effects of different carbon sources\* on *A. azollae* nitrogenase activity (nmoles  $C_2H_4$  (mg chlorophyll) $^{-1}$ .min $^{-1}$ ) in the dark under aerobic ( $O_2 = 20\%$ ) and microaerophilic ( $O_2 = 1\%$ ) conditions. (Temperature: 20 °C; pH: 7,0). [Each value is a mean of four replicates]

Oxygen concentration	Carbon source			Control (autotrophic)
	Glucose	Fructose	Sucrose	
1 %	60,8	151,2	61,4	97,3
20 %	50,7	100,8	50,2	81,9

[\* Final concentration was 0,2% for each carbon source used]

#### 7.4.4 Recombination of the components

All attempts to reconstitute the symbiotic association by combining the isolated fern and algal components were unsuccessful. In the case where alga-free plants were sprayed with a suspension of *A. azollae* filaments each day, a few algal filaments survived as epiphytes between the leaf bases. No algal cells were incorporated within the leaf cavities when alga-free plants were grown with either symbiotic plants or with cultured *A. azollae*. Alga-free plants were very brittle and fragmented easily during the shaking treatment necessary to prevent sedimentation of the algal filaments. The alga-free plants also fragmented and died when cultures were bubbled instead of shaken to maintain an algal suspension. None of the alga-free megaspores became "reinfected" with *A. azolla*, and all megaspores died within two weeks.

Injection of a suspension of *A. azollae* into the fern's apical meristematic region caused rapid necrosis and death of the stem apex. The rest of the stem was unaffected and lateral branches grew normally. *Anabaena azollae* cells injected into the space between upper and lower leaf lobes were unable to enter the leaf cavity and remained between the leaf lobes for two to three days before becoming desiccated and dying. When these two injection treatments were repeated with freshly isolated (but non-axenic) *A. azollae*, the results were also uniformly negative. Again, stem apices became necrotic and algal cells between the leaf bases did not enter the leaf cavities.

#### 7.5 DISCUSSION

The successful isolation and culture of the component organisms in the *Azolla filiculoides*-*Anabaena azollae* symbiosis reported in this study is an important step in understanding the interactions between the organisms and the mechanisms of the symbiotic association.

The use of surface-sterilized material and sterile micromanipulation techniques ensured that only algae from *A. filiculoides* leaf cavities were isolated in this investigation. Though this procedure is extremely tedious, the possibility of epiphytic algal contamination is eliminated. Several earlier reports of successful *A. azollae* isolation and growth (Vouk and Wellisch, 1931; Huneke, 1933; Shen, 1960; Venkataraman, 1962) are suspect because non-sterile *Azolla* material was used.

The successful process for isolating *A. azollae* from *A. filiculoides* reported here has selected for autotrophic nitrogen-fixing strains capable of *in vitro* growth. These cell forms reportedly form only a small proportion of the total *A. azollae* population within an *Azolla* frond (Hill, 1975, 1977; Newton and Herman, 1979). The low number of successful isolates obtained here suggests that most of the algal population within *A. filiculoides* may be incapable of sustained growth. The varied morphology of *A. azollae* cells within *A. filiculoides* leaf cavities, often consisting of moribund and thick-walled resting cells, especially in the older leaves (Section 4.4.4), supports this hypothesis. Further support is provided by the recent report that massive algal inocula ( $> 10^7$  cells/ml) were necessary for successful isolation of *A. azollae* (Newton and Herman,

1979). Several authors have reported that *A. azollae* can be isolated from *Azolla* plants but that the alga did not survive longer than a few days in culture (Shields and Durrell, 1964; Lang, 1965; Peters, 1975, 1976; Hill, 1975; Singh, 1977a). This failure, ascribed to bacterial contamination or the lack of suitable media, has been a major obstacle to elucidating the mechanisms of the symbiosis.

During this study, most attempts to isolate and culture *A. azollae* were unsuccessful due to excessive bacterial contamination. The leaf cavities of *A. filiculoides* contain numerous bacteria (Section 4.4.1), a feature common to other species of *Azolla* (Venkataraman, 1962; Weiringa, 1968; Peters and Mayne, 1974b). These bacteria are predominantly aerobes (Peters and Mayne, 1974b) and Fisher *et al.* (1980) have shown them to be mainly coryneform bacteria. In the isolation procedure employed here, all non-sporing bacteria were killed by heat treatment as advocated by Weiringa (1968). Residual slow-growing, spore-forming (or heat-resistant) bacterial strains which survived this treatment were eliminated by repeated streaking of sub-cultures on solid media.

In this investigation, the N-free modification of the inorganic BG-11 medium designed by Stanier *et al.* (1971) was found to be suitable for the isolation and culture of *A. azollae*. Newton and Herman (1979) also used an N-free variation of this medium for their isolation of *A. azollae* and observed that the provision of an organic carbon source in the isolation medium led to overwhelming bacterial growth and the subsequent death of the algal cells. This feature was also cited by Peters and Mayne (1974b) as the main reason for their lack of success in isolating *A. azollae*. Leaf squash techniques and those involving maceration and centrifugation of whole *Azolla* fronds which involve the transfer of organic compounds to the isolation medium can therefore be expected to have a high failure rate due to bacterial contamination.

Successful removal of *A. azollae* from the leaf cavities of *A. filiculoides* with antibiotic treatments has confirmed the earlier studies of Nickell (1958), Walmsley *et al.* (1973) and Peters and Mayne (1974a, b). The presence of bacteria (observed by light microscope, phase-contrast illumination) within the leaf cavities of the alga-free fern indicates that the bacteria had either recolonized the mucilage or were apparently

unaffected by the antibiotic treatments, in contrast to the observations reported by Nickell (1958). The alga-free fern requires a source of combined nitrogen ( $\text{NO}_3\text{-N}$  or  $\text{NH}_4\text{-N}$ ) in the medium and was maintained in culture for over 18 months without excessive bacterial growth. However, the possibility that the antibiotic treatments alter some aspect of the fern's metabolism cannot be excluded.

The reports by Huneke (1933), Schæede (1947) and Hill (1975) that alga-free ferns could be obtained by subjecting *Azolla* plants to sudden changes in their environment (e.g. light intensity and temperature) were not confirmed in this study. This feature points to the possibility that the endosymbionts found in different species and varieties of *Azolla* may in fact be physiologically distinct varieties. This might account for the different degrees of success in isolating and culturing *A. azollae* that have been reported (Moore, 1969; Lumpkin and Plucknett, 1980). However, the results of this study indicate that *A. azollae* and the alga-free *A. filiculoides* plants do have different growth optima. Therefore, this type of approach, i.e. providing an environment which effectively favours one of the partners in the symbiosis, may well work under certain conditions and deserves further investigation. In this regard, the controlled environment studies have indicated that the fern and algal components are possibly more sensitive to environmental extremes than the symbiotic association since optimal fern  $\overline{\text{RGR}}$  values and algal nitrogenase activity are only recorded within a rather narrow range of each environmental variable tested. In addition, *A. azollae* often tolerates a much wider range of conditions than the alga-free fern. Under all conditions tested, the  $\overline{\text{RGR}}$  values of the alga-free fern are considerably lower than those measured for the intact association.

Both the  $\overline{\text{RGR}}$  of the alga-free fern and nitrogenase activity by *A. azollae* are dependent on temperature. When day and night temperatures are kept constant, the temperature range permitting the highest  $\overline{\text{RGR}}$  and nitrogenase activity values (20-25 °C) is identical to that found for the intact association at low light intensities. However, the total temperature range tolerated by the alga-free fern is much narrower than that recorded for the intact association (Section 5.3.2) or *A. azollae*. Thus, removal of the alga results in a lower temperature tolerance in the alga-free fern.

When the isolated organisms are grown with a 10 °C temperature differential between nocturnal and diurnal temperatures, their respective  $\overline{\text{RGR}}$  and nitrogenase activity values are similar to those recorded under constant temperatures. However, both *A. azollae* and the alga-free fern tolerate higher diurnal and lower nocturnal temperatures. Rodgers (1978) recorded a similar temperature range for nitrogenase activity in both the isolated *Nostoc* and the intact *Nostoc-Blasia pusilla* association.

In isolated *A. azollae*, nitrogenase activity is low at pH levels below 7,0. This feature is shared by many free-living heterocystous blue-green algae which rarely grow or fix nitrogen at pH levels below 6,0 (Jurgensen and Davey, 1968; Dooley and Houghton, 1973) and endophytic *Nostoc* isolated from *Blasia thalli* (Rodgers, 1978). However, in common with the *Nostoc-Blasia* association, *A. azollae* in the intact *Azolla* association can reduce acetylene over a wide pH range. Therefore, within the leaf cavities of the intact *Azolla-Anabaena* association, the algal colonies must be buffered from the effects of low external pH. Rodgers (1978) has postulated that the *Nostoc* cells in the *Nostoc-Blasia* association might be buffered from the effects of low external pH either by the mucilage in the algal cavities or by the cells of the liverwort host surrounding the cavities. A similar situation has been reported for the moss *Sphagnum*, where intracellular *Nostoc* filaments in the leaves are protected from low external pH (Granhall and Selander, 1973). This protective buffering effect is beneficial to the endophytic alga, and to the host plant, since it allows the alga to fix nitrogen over a wider range of external pH.

The sensitivity of both *A. azollae* and the alga-free fern to high nutrient concentrations ( $> 200 \text{ mg.l}^{-1}$ ) is similar to that exhibited by the intact fern-alga association. This sensitivity appears to be due to an osmotic effect since the algal cells become discoloured, shrunken and die whilst the alga-free ferns appear shriveled and chlorotic.

The successful culture of sterilized (*Anabaena*-free) apical meristems of *Azolla* reported by Duckett *et al.* (1975a) was not confirmed in this study. Theoretically, the use of such tissue-culture techniques should be entirely feasible and would thus eliminate the possible side-effects of treatments with antibiotics. Indeed, Duckett *et al.* (1975a) indicated that

their technique produced "vigorous vegetative plants within two to three weeks". In the present investigation, the sterilization procedure could well have been too severe, since the treated apices became necrotic and died. Microscopic examination of alga-free megaspores which had failed to germinate indicate that this failure was due to mechanical damage of the megaspore. Despite the lack of success in this study, the above two methods show promise as possible methods of obtaining alga-free *Azolla* plants and should be further investigated. Ideally, these techniques would produce healthy, vigorously-growing plants whose metabolism has not been affected by chemical treatments. However, in order to produce axenic (bacteria-free) cultures of alga-free *Azolla* plants, some method would have to be devised to remove the bacteria from the fern's leaf cavities.

The isolated *A. azollae* is capable of growth on media lacking a source of combined nitrogen and is also able rapidly <sup>to</sup> reduce acetylene to ethylene whereas the alga-free fern requires a source of combined nitrogen for growth and is unable to reduce acetylene. Sub-cultures of the contaminating bacteria, isolated from *A. filiculoides* leaf cavities, are also unable to reduce acetylene. These observations further confirm that *A. azollae* is the nitrogen-fixing organism within the symbiotic association. An earlier report (Bottomly, 1920) that bacteria are responsible for nitrogen fixation in the symbiosis is possibly due to contamination and a lack of knowledge regarding algal nitrogen fixation.

The results from this study provide evidence that *A. azollae* is capable of heterotrophic growth in the dark and confirm the observations of Newton and Herman (1979). In agreement with the results presented here, these two authors showed negligible dark heterotrophic growth on glucose. Earlier workers have demonstrated dark heterotrophic growth and nitrogen fixation by isolated endosymbiotic blue-green algae (Hoare *et al.*, 1971; Fogg *et al.*, 1973; Stewart and Rodgers, 1978). The results from this study also infer that nitrogenase activity in *A. azollae* might be a preferentially heterotrophic process which depends on the fern for supplies of carbohydrate. This hypothesis is consistent with the observations of Peters and Mayne (1974b) and the data shown previously (Section 5.3.1), that dark nitrogenase activity in the intact association is dependent on previous light treatments. During this investigation, a variety of nitrogen forms

were tested as to their suitability for sustaining the growth of the alga-free fern and *A. azollae*. These results are reported in detail in the next chapter of this thesis.

Oxygen has been shown to inhibit nitrogen fixation both in free-living blue-green algae (Stewart and Pearson, 1970) and endophytic *A. azollae* in the intact association (Peters and Mayne, 1974b). In the present study, the nitrogenase activity of isolated *A. azollae* filaments increases under microaerophilic conditions, confirming the sensitivity of the algal nitrogenase to  $O_2$ . However, the mechanisms whereby *Azolla* could maintain low  $O_2$  tensions within its leaf cavities are not known at present.

Since photosynthesis is either directly or indirectly the source of reductant and ATP required by nitrogenase in blue-green algae (Stewart, 1974), light intensity affects both the intact *Azolla-Anabaena* association and isolated *A. azollae*. In the laboratory, the optimal light intensity for nitrogenase activity in *A. azollae* is lower than that required to saturate nitrogenase activity in the intact association (Section 5.3.2). At light intensities approaching full daylight, *A. azollae* cells become discoloured and soon die, possibly due to photo-oxidation. Thus, in the intact association, the *Azolla* leaf cavity provides a degree of physical protection for the alga from high light intensities. This would be especially important if *A. Azolla* is as susceptible to photo-oxidation as several other blue-green algae (Abelovitch and Shilo, 1972). A similar proposal has been put forward to account for the protective effects of *Blasia* thalli on their endophytic blue-green algae (Rodgers, 1978). The original suggestion by Peters and Mayne (1974a), that the fern leaf may modify the quality of light incident on the alga in the intact association, is therefore supported by results obtained during this study. The enhanced *A. azollae* nitrogenase activity under green light, as compared with either red or blue light, corresponds with the results obtained using the intact association. This suggests that the chlorophyllous upper palisade layer of the *Azolla* dorsal leaf lobe may act as a green filter and that *A. azollae* is chromatically adapted (De Marsac, 1977) to utilize this light wavelength. Ray *et al.* (1979) and Tyagi *et al.* (1981) have recently shown that the pigmentation of *A. azollae* complements that of the fern, absorbing

light in that portion of the visible spectrum where absorption by the fern pigments is minimal. They also showed that endophytic *A. azollae* contained several phycobiliproteins (notably phycocyanin and phycoerythrin) whose light absorption maxima are ideally suited to utilize this incident light and thereby supply energy for nitrogen fixation. However, in this context, it must be borne in mind that *A. azollae* populations within *Azolla* leaf lobe cavities have very high heterocyst frequencies. Fogg *et al.* (1973), who worked on free-living species of blue-green algae, reported that heterocysts lack photosystem II and that virtually all of the reduced carbon compounds and energy required for nitrogen fixation were provided by the vegetative cells. The recent studies of Ray *et al.* (1979) and Tyagi *et al.* (1981) have confirmed that the same situation exists in *A. azollae*. However, these authors point out that the relatively low numbers of vegetative cells would probably be unable to supply all of the energy and carbon skeletons required by the heterocysts to maintain the high rates of nitrogenase activity that have been recorded. Though this evidence is indirect, it does lend weight to the hypothesis that nitrogen fixation in *A. azollae* may be preferentially a heterotrophic process.

Previous attempts to recombine *A. azollae* and alga-free *Azolla* have always been unsuccessful (Huneke, 1933; Bortels, 1940). In this study, attempts to reconstitute the symbiotic association by combining the isolated fern and algal components, and thereby fulfil all of Koch's postulates, were also unsuccessful. This failure could be due to either one or a combination of the following reasons:

- (a) an unsuitable method of recombining the components,
- (b) the isolated alga is not in fact *A. azollae*,
- (c) the isolated alga is *A. azollae*, but *in vitro* culture has caused some physiological change to the alga, and
- (d) antibiotic treatments have altered the alga-free fern's physiology.

Successful recombination of the two components entails the incorporation of the alga into the fern's leaf cavities, followed by the resumption of

symbiotic growth. Because alga-free ferns are compact with closely-adpressed leaves, this is extremely difficult to achieve, particularly in the case of older leaves, without damaging the fern host. On morphological grounds, if the fern's apical meristem can be "infected" with the alga, normal fern leaf development will automatically incorporate algal cells into the leaf cavity. Since the fern's apical meristem curves upwards, away from the water surface, the failure of dual liquid culture attempts are not unexpected. Micromanipulation and micro-injection techniques to infect the fern apex with algal cells are therefore the most promising methods to use. The consistent failure of these techniques in this study, as well as those methods employing surface sprays of algal cells, point to incorrect methodology. These techniques should therefore be refined before future recombinations of the fern and algal components are attempted.

Several authors (e.g. Bortels, 1940; Moore, 1969; Lumpkin and Plucknett, 1980) have postulated that, due to poor techniques, the isolated alga may not have been *A. azollae*. In this study, sterile micromanipulation techniques were used to isolate the alga directly from the fern's leaf cavities. This feature, combined with the morphological evidence (cell dimensions, heterocyst frequencies) and physiological evidence (heterotrophic ability, patterns of nitrogenase activity in response to environmental stimuli), strongly suggest that the alga isolated from *A. filiculoides* in this study is indeed *A. azollae*. However, the ultimate identification of the alga as an *A. azollae* strain will require its successful re-introduction into alga-free *Azolla* plants and the resumption of symbiotic growth.

The nature of the isolation process used in this study selected for nitrogen-fixing autotrophic strains of *A. azollae* capable of growth *in vitro*. This feature, combined with the very low number of successful isolates, indicates that only a very small proportion of the original population could survive and grow under these conditions. The morphological changes demonstrated by isolated *A. azollae* during the first few months of *in vitro* culture imply simultaneous alterations in physiological capabilities (Ray *et al.*, 1979). The temporal changes in cell dimensions

follow a pattern of enlargement similar to that recorded during natural aging of *A. azollae* in the intact association (Section 4.4.4). Hill (1975, 1977) has shown that these morphological changes accompany changes in nitrogenase activity and metabolism. However, the decreased heterocyst frequency shown in aging cultures of isolated *A. azollae* may be a response to conditions in the external medium. Therefore, the possibility that the isolated *A. azollae* is physiologically distinct from the algae within the symbiosis cannot be excluded.

Because of the antibiotic treatments used in removing *A. azollae* from the fern, it is quite possible that some aspect of the alga-free fern's physiology was altered. Although microscopic examination revealed no noticeable anatomical changes in the fern, the removal of the alga clearly has a pronounced effect on the fern's metabolism since even under optimal conditions on a combined nitrogen source the growth rate of the alga-free plant is much lower than that of the intact association. Rodgers and Stewart (1977) and Stewart (1978) have clearly demonstrated the highly specific nature of symbiotic associations between blue-green algae and eukaryotic organisms. The mechanisms of this specificity are unknown at present, but one of the factors involved might be the presence of lectins (sugar-binding proteins). These lectins and their glycoconjugate receptors have been implicated in cellular recognition and adhesion in Rhizobia-legume interactions and lichen symbioses (Schmidt, 1979; Lockhart, Rowell and Stewart, 1978). More recently, Kobilier, Cohen-Sharon and Tel-Or (1981) have shown that isolated *A. azollae* possesses a lectin loosely bound to the cell and that the fern contains a highly specific endogenous receptor. Further investigation is needed in this field to determine whether or not systematic differences exist between *A. azollae* strains isolated from different species of *Azolla*.

The *Azolla-Anabaena* association can be called a true symbiosis since both of the partners derive some degree of benefit from the association. Evidence gained from this and other studies indicates that what at first might appear to be a rather simple association is, in fact, highly complex, with morphological, physiological and biochemical modifications and recognition mechanisms, all being involved as the association develops. Thus, the development of processes to successfully isolate and

recombine the two organisms is an important step in the study of the symbiotic association. In this study it was found to be relatively easy to produce alga-free plants of *A. filiculoides* but considerably more difficult to isolate *A. azollae*. However, despite the ability of the fern and the alga to exist separately in culture, it is obvious that for normal growth, the fern relies heavily on the presence of the alga. Therefore, the controversy that exists about the fern-alga association and the inter-relationships between the two organisms will continue until the two organisms have been successfully recombined *in vitro*.

## CHAPTER 8

### NITROGEN ASSIMILATION AND METABOLISM

#### 8.1 INTRODUCTION

In the *Azolla-Anabaena azollae* symbiosis, nitrogen plays a fundamental role in both metabolism and growth (Ashton and Walmsley, 1976). Because of the nature of the symbiotic association, *Azolla* plants are able to utilize two major pathways of nitrogen assimilation (Holst and Yopp, 1979b). These are absorption of combined nitrogen sources from an aqueous medium by the *Azolla* host and fixation of atmospheric nitrogen by the *Anabaena* symbiont. Both of these processes can be utilized independently to fulfil the total nitrogen requirements of the symbiotic association (Ashton and Walmsley, 1976). However, the total nitrogen content assimilated by the symbiosis under natural conditions would normally be the sum of the functioning of these two processes.

Although *Azolla* plants may take up combined nitrogen in a variety of forms, the main nitrogen sources absorbed under natural conditions are ammonium and nitrate ions. Both of these ionic nitrogen forms are readily absorbed, though high concentrations of ammonium ions can become toxic and inhibit plant growth (Moore, 1969). Within the plant, absorbed nitrogen can only be incorporated into organic forms when it is in the reduced form, ammonium (Haynes and Goh, 1978). Thus, ammonium ions may be incorporated directly while nitrate ions must first be reduced to ammonium before assimilation can take place. This reduction of nitrate occurs in two steps and is catalysed by the enzymes nitrate reductase and nitrite reductase (Haynes and Goh, 1978).

Fixation of atmospheric nitrogen via the algal symbiont is accomplished by the nitrogenase enzyme complex and, as in the nitrate-nitrite reductase system, the ultimate product is ammonia (Moore, 1969). Feedback inhibition by this product has been noted for both enzyme systems (Peters *et al.*, 1977). Therefore, the functioning of one system can inhibit the activity of the enzyme(s) of the other system.

Several well-documented laboratory studies have separately demonstrated the capability of the phycobiont to fix atmospheric nitrogen and the presence of the nitrate-nitrite reductase system in the *Azolla* host (e.g. Peters *et al.*, 1977; Holst and Yopp, 1979b). However, conclusions as to the inter-relationships of these two assimilatory pathways have mostly been based on studies using unnaturally high concentrations ( $\text{mg} \cdot \text{l}^{-1}$ ) of combined nitrogen (Moore, 1969). The applicability of these findings to the conditions pertaining to natural populations of *Azolla* is often tenuous.

The field and laboratory studies carried out during this investigation were designed to:

- (a) demonstrate the presence of the two main nitrogen assimilation pathways in the *Azolla filiculoides*-*Anabaena azollae* symbiosis, and their location in the individual symbionts,
- (b) elucidate the effects of the major environmental variables on the two processes and evaluate the efficiency of each process as a means of providing the symbiosis with its nitrogen requirements, and
- (c) assess the significance of the two nitrogen assimilation pathways to natural populations of *Azolla filiculoides*.

## 8.2 Literature review

Since the endosymbiotic alga *Anabaena azollae* has been found in all *Azolla* species (Lumpkin and Plucknett, 1980), the nitrogen metabolism and the inter-relationships between fern and alga are probably very similar in each species. Therefore, information derived from other *Azolla* species can contribute to our understanding of the situation in *A. filiculoides*. The *Azolla*-*Anabaena* association can obtain nitrogen by nitrogen fixation, by absorption from an aqueous medium or by a combination of the two processes (Peters *et al.*, 1976, 1977). Despite a decrease in nitrogenase activity when grown with a source of combined nitrogen (Walmsley *et al.*, 1973; Peters and Mayne, 1974b), *Azolla* plants do not lose their ability to fix atmospheric nitrogen even after six to seven months growth on a medium containing combined nitrogen (Peters and Mayne, 1974b). However,

under ideal laboratory conditions, *Azolla* plants register maximum values of  $\overline{\text{RGR}}$  and nitrogenase activity on a nitrogen-free medium (Peters *et al.*, 1976, 1977). Therefore, *Azolla* can more efficiently fulfil its nitrogen requirements by fixing atmospheric nitrogen.

Indirect evidence for the fixation of nitrogen by symbiotic *Azolla* plants has been provided by experiments in which the intact association was grown in nitrogen-free nutrient solutions and increases in plant nitrogen content determined by Kjeldahl analysis (Oes, 1913; Mameli and Pollacci, 1914; Bortels, 1940; Saubert, 1949; Johnson *et al.*, 1966; Moore, 1969; Twyman and Ashton, 1972). Definite proof that *A. azollae* is the agent of nitrogen fixation in the association has only recently been provided, using the acetylene reduction assay (Walmsley *et al.*, 1973; Peters and Mayne, 1974b; Peters, 1975, 1976; Newton, 1976). Both the fern-alga association and the isolated alga are able to reduce acetylene, whereas the alga-free fern can not, nor can it sustain growth on a nitrogen-free medium (Walmsley *et al.*, 1973; Peters and Mayne, 1974b; Peters, 1975, 1976; Newton, 1976). Subsequent studies utilizing  $^{15}\text{N}_2$  have confirmed and amplified these findings (Peters *et al.*, 1977; Kaplan and Peters, 1981).

The implication of the algal symbiont as the actual agent of nitrogen fixation implies the transfer of considerable quantities of fixed nitrogen between the two organisms. In the intact association, the development of the alga parallels that of the fern (Konar and Kapoor, 1972; Hill, 1977; Peters *et al.*, 1978). Nitrogenase activity is low in algal cells at the fern apex, increasing rapidly with increasing algal cell age and then declining as the cells senesced (Hill, 1977; Peters, Ray *et al.*, 1980; Kaplan and Peters, 1981). Sequential analysis of leaf nitrogen content, combined with acetylene reduction assays, indicates that nitrogen is transferred both to the senescent algal cells in older *Azolla* leaves and to the undifferentiated algal cells at the fern apex (Hill, 1977; Kaplan and Peters, 1981). Studies with  $^{15}\text{N}_2$  have shown that the endophyte isolated from *Azolla* leaves releases up to 50 % of its fixed nitrogen as ammonia (Newton and Cavins, 1976; Peters, 1977; Peters, Ray *et al.*, 1980). Within the association, high levels of intra-cellular (?intra-cavity) ammonia are found (Newton and Cavins, 1976) and fixed  $^{15}\text{N}_2$  is rapidly assimilated and incorporated into amino acids and protein

(Peters *et al.*, 1979). Further proof has been provided by the finding that almost all of the activities of the major aminating enzymes glutamate dehydrogenase and glutamine synthetase can be attributed to the *Azolla* host (Ray *et al.*, 1979). This is in accord with the suggestion (Stewart, 1978; Haselkorn, 1978) that in plant-cyanophyte associations the plant modifies the cyanophyte's ammonia assimilating pathway by producing substances which inhibit either glutamine synthetase activity or synthesis in the endophyte. Thus, in the *Azolla-Anabaena* association, it is possible that the ammonia released by the endophyte in the cavities of mature leaves is being transported, or that the ammonia is metabolized to glutamine by the *Azolla* glutamine synthetase and then transported, or that both processes are occurring (Kaplan and Peters, 1981).

Moore (1969) noted that the published data of many workers on the growth of *Azolla* supplied with combined nitrogen were inconsistent. Oes (1913) demonstrated poor growth of *Azolla* (but higher plant nitrogen content) if  $\text{NH}_4\text{-N}$  in solution or in the atmosphere were supplied. However, Bortels (1940) and Tuzimura, Ikeda and Tukamoto (1957) [cited in Moore (1969)] reported that the addition of low concentrations of either  $\text{NH}_4\text{-N}$  or  $\text{NO}_3\text{-N}$  accelerated growth. Subudhi and Singh (1979b) showed that high concentrations ( $56 \text{ mg N.l}^{-1}$ ) of both  $\text{NH}_4\text{-N}$  and  $\text{NO}_3\text{-N}$  decrease growth rates, though the ammonia-grown plants had higher nitrogen and soluble sugar contents. Brotonegoro and Abdulkadir (1976) reported similar effects and noted that their cultures of *A. pinnata* took 24 hours before the adverse effects of combined nitrogen additions were manifest. Yatazawa *et al.* (1980) found that both growth and nitrogenase activity decreased as progressively higher  $\text{NH}_4\text{-N}$  concentrations were added to their culture media. LeVan and Sobachkin (1963) and Singh (1977a) also noted that the addition of nitrogenous fertilizers to tank-grown *A. pinnata* plants decreased the yield. In contrast, high concentrations (greater than  $50 \text{ mg N.l}^{-1}$ ) of  $\text{NO}_3\text{-N}$  or  $\text{NH}_2\text{CONH}_2$  in the growth medium did not change the growth rate of *A. caroliniana* (Peters, 1975). However, high concentrations ( $> 50 \text{ mg.l}^{-1}$ ) of either  $\text{NO}_3\text{-N}$  or  $\text{NH}_2\text{CONH}_2$  have been shown to cause a 20-30 % decrease in aerobic-light nitrogenase activity in *A. caroliniana* and *A. filiculoides* (Walmsley *et al.*, 1973; Peters and Mayne, 1974b; Newton, 1976). The same concentrations of  $\text{NH}_4\text{-N}$  caused a 90 % decrease in aerobic-light nitrogenase activity (Peters and Mayne, 1974b). In an atmosphere free of nitrogen ( $\text{Ar} - \text{CO}_2 - \text{O}_2$ ), *A. caroliniana*

plants grown on nitrogen-free media or with  $\text{NH}_4\text{-N}$  became necrotic within 15 days, while plants supplied with  $\text{NO}_3\text{-N}$  or  $\text{NH}_2\text{CONH}_2$  showed the same nitrogenase activity as those recorded under aerobic conditions and remained green and healthy (Peters and Mayne, 1974b).

Since the algal symbiont is not in direct contact with either the external environment or the vascular system of the plant, it is unlikely that  $\text{NO}_3\text{-N}$  or  $\text{NO}_2\text{N}$ , as such, reach the alga in the leaf cavity, but are rather metabolized by the fern (Peters, 1977; Peters *et al.*, 1978). This is also indicated by both the retention of nitrogenase activity in the symbiont and the absence of an effect of nitrate upon the heterocyst frequency of the symbiont in the intact association when grown on media containing  $\text{NO}_3\text{-N}$  (Hill, 1975; Peters, 1977). The ability of the *Azolla-Anabaena* symbiosis to grow on the combined nitrogen source,  $\text{NO}_3\text{-N}$ , implies the existence of nitrate and nitrite reductase enzymes that are necessary to reduce  $\text{NO}_3\text{-N}$  to  $\text{NH}_4\text{-N}$ . However, conclusive evidence that the fern contains these enzymes has only recently been provided for *A. mexicana* by Holst and Yopp (1979b, c).

Conflicting reports have been published on the ability of different *Azolla* species to release nitrogenous compounds into their aquatic environment. Saubert (1949) reported that 2 % of the nitrogen assimilated by *A. pinnata* was released, while several Chinese workers reported that a variety of *A. pinnata* released between 14 and 21 % of its fixed nitrogen (Lumpkin and Plucknett, 1980). Despite the speculation by Talley *et al.* (1977) that *A. mexicana* might release fixed nitrogen, this was not demonstrated. Peters (1977) also found no nitrogenous compounds in a culture solution where *A. caroliniana* had been grown. However, it is possible that the release of fixed nitrogen to the medium may only occur when the plants in lower senescent layers of an *Azolla* mat begin to decay (Gopal, 1967).

### 8.3 MATERIALS AND METHODS

#### 8.3.1 Plant and algal material

Mature *A. filiculoides* plants were collected in the field, transported to the laboratory and grown in large constant environment rooms (Section 2.5.2.1). Prior to use, the plants were surface-sterilized to remove

epiphytic contaminants (Section 2.5.2.2) and grown in 500 ml capacity Erlenmeyer flasks (Section 2.5.2.2) containing 300 ml of the appropriate nutrient medium.

Alga-free *Azolla* fronds and cultures of isolated *Anabaena azollae* were obtained as described previously (Sections 7.3.1 and 7.3.2). Due to the scarcity of material and slow *in vitro* growth rates, investigations were conducted 6 to 9 weeks after isolation of the respective components, when sufficient new growth had occurred.

### 8.3.2 Growth conditions

Several series of experiments were carried out in the constant environment rooms to investigate the effects of a wide range of environmental variables on the pathways of nitrogen assimilation. The variables investigated were: photoperiod, light intensity, light quality, constant temperature, diel temperature fluctuations, pH and osmotic stress. In each experiment, one environmental variable was tested at several different levels while the other variables were held constant. This was accomplished automatically by adjusting the appropriate control selectors on the constant environment rooms. Prior to each experiment all plant material was acclimatized for at least 10 days under the particular environmental conditions under investigation. In those investigations involving the isolated fern and algal components, only four replicates of each treatment were used due to the scarcity of material. Eight replicates were used in those experiments employing symbiotic *A. filiculoides* material. Growth rate was measured as described in Section 2.5.2.2 and nitrogenase activity was assessed with the acetylene reduction assay (Section 5.2.1.1).

### 8.3.3 Nutrient media

Symbiotic *A. filiculoides* plants were grown on Azolla-I medium supplemented, where necessary, with different forms of combined nitrogen administered either as  $\text{NaNO}_3$ ,  $\text{NaNO}_2$  or  $\text{NH}_4\text{Cl}$ . The nitrogen concentrations used in each experiment are detailed in the results (Section 8.4). A fourth source of combined nitrogen, urea ( $\text{NH}_2\text{CONH}_2$ ), was also tested. All of these nutrients were of analytical grade and were not further purified. During each experiment, media were replaced daily by siphoning.

*Anabaena azollae* was routinely cultured in N-free BG-11 medium (Section 7.3.1), while alga-free *A. filiculoides* plants were grown in Azolla-I(+N) medium (Section 7.3.2). The ability of four different forms of combined nitrogen (listed above) to support growth of the isolated components was also tested.

Atmospheric conditions within culture flasks were varied by altering the ratio of several component gases (Ar, N<sub>2</sub>, O<sub>2</sub>, CO<sub>2</sub>) with a Matheson model 665 gas proportioner. These gases were of the highest purity available and were obtained from African Oxygen Ltd, Port Elizabeth.

#### 8.3.4 Assays for nitrate reductase and nitrite reductase

The activities of the enzymes nitrate reductase and nitrite reductase were measured using a modification of the *in vivo* assay described by Klepper, Flesher and Hageman (1971) and Hageman and Hucklesby (1971). This method relies on the gentle vacuum infiltration of the assay medium into the tissue (or organ) under investigation and the spectrophotometric analysis of changes in the composition of the assay medium with time. The assay procedures for both nitrate reductase and nitrite reductase are essentially the same, analysing for the appearance and disappearance of NO<sub>2</sub><sup>=</sup> ions respectively. Initial experiments yielded very erratic values for rates of enzyme activity. This had also been noted for the *in vivo* assay by Ferrari and Varner (1970), who indicated that this was due to differential permeability of the tissues used. These authors recommended the use of 1,5 % (v/v) ethanol in both the infiltration and assay media to alleviate permeability barriers. When tested, this procedure resulted in far less variation in enzyme activity within treatments and was adopted for routine use.

The nitrate reductase assay involves measuring the rate of production of NO<sub>2</sub>-N (Kumada, 1953). Normally, the NO<sub>2</sub>-N produced is reduced in the tissue by nitrite reductase, using photoreductant as energy source. Theoretically, if the assay is carried out in the light, no NO<sub>2</sub>-N production would occur since nitrate reductase activity is normally equal to or slightly less than nitrite reductase activity (Randall, 1969; Klepper *et al.*, 1971). Thus, in order to reduce this *in vivo* reduction of NO<sub>2</sub>-N to a minimum, the nitrate reductase assay is performed in the dark

after a short (15 minute) preincubation in the dark (Randall, 1969; Hageman and Hucklesby, 1971).

The plant material to be assayed for nitrate reductase activity was washed with sterile distilled water and gently vacuum-infiltrated (40 mm Hg) with an assay medium for 20 seconds in dim light. This assay medium consisted of 1,5 % (v/v) ethanol in a 1:1 mixture of 0,1 M  $\text{KNO}_3$  and 0,1 M phosphate buffer (pH 7,5). After infiltration, approximately 0,5 g of plant material was then transferred to a test tube containing 10 ml of fresh assay medium, and placed in the dark at room temperature (approximately 22 °C). After 10 minutes, the contents of the tube were gently vortexed and a 1 ml aliquot removed to a test tube containing 2 ml of a 1 % (m/v) solution of sulphanilamide in 3 N HCl. After a further 30 to 60 minutes, the process was repeated and an additional aliquot removed for analysis. Two millilitres of 0,01 % (m/v) N-(N-naphthyl) ethylene diamine-HCl solution in water were then added with mixing to each tube and the colour allowed to develop for 30-35 minutes. After colour development, absorption at 540 nm was measured with a Pye Unicam SP-8000 spectrophotometer and the  $\text{NO}_2\text{-N}$  concentration calculated from a calibration curve prepared with analytical grade  $\text{NaNO}_2$ .

The assay procedure for nitrite reductase was virtually identical to that for nitrate reductase except that  $300 \mu\text{g} \cdot \text{l}^{-1} \text{NO}_2\text{-N}$  (as  $\text{NaNO}_2$ ) was substituted for the  $\text{NO}_3\text{-N}$  in both the infiltration and assay media. Here, the enzyme activity was based on the rate of disappearance of  $\text{NO}_2^-$  ions. Those assays requiring a light source were conducted in a constant environment room under a light intensity of 5 kLux.

#### 8.3.5 The effects of mat formation on nitrogen assimilation

In order to assess the influence of mat formation on rates of nitrogen assimilation, *A. filiculoides* plants were grown in three shallow asbestos trays (7 cm depth,  $0,21 \text{ m}^2$  surface area) on N-free Azolla-I medium. Each tray contained 10,5 l of nutrient medium which was sufficient to maintain a depth of 5 cm. Each tray was inoculated with 2,0 g of acclimatized *A. filiculoides* plants at the start of the experiment and the plants were allowed to grow for a period of 50 days. Every five days triplicate samples of *A. filiculoides* plants were removed from each tray and their

nitrogenase activity and nitrate reductase activity measured as described in Sections 5.2.1.1 and 8.3.3, respectively. At the same time, a 150 ml sample of the nutrient solution in each tray was removed for chemical analysis of the nitrogen forms present in the medium. All losses in the volume of medium due to evaporation and the withdrawal of samples for analysis were replaced with fresh Azolla-I medium.

#### 8.4 RESULTS

##### 8.4.1 The effects of different nitrogen sources on growth and nitrogen assimilation

The intact *Azolla filiculoides*-*Anabaena azollae* association was able to sustain growth for at least 90 days on a variety of combined nitrogen sources (Table 52). Higher  $\overline{\text{RGR}}$  values ( $> 0,262 \text{ g.g}^{-1} \cdot \text{day}^{-1}$ ) were recorded when the symbiosis was grown with atmospheric  $\text{N}_2$  as the sole nitrogen source. Where necessary, a mixture of 79,9 % Ar: 20 %  $\text{O}_2$ :0,1 %  $\text{CO}_2$  at a flow rate of  $30 \text{ ml} \cdot \text{min}^{-1}$  was used to maintain an N-free gas phase in those cultures containing combined nitrogen. In the complete absence of atmospheric nitrogen or a source of combined nitrogen, all *Azolla* fronds became necrotic and died within 18 days. When the plants were supplied with combined nitrogen,  $\overline{\text{RGR}}$  values increased with increasing nitrogen concentration up to  $1,0 \text{ mmol}_\lambda$ . Low  $\overline{\text{RGR}}$  values ( $< 0,169 \text{ g.g}^{-1} \cdot \text{day}^{-1}$ ) were recorded at  $10,0 \text{ mmol}_\lambda$  for all four combined nitrogen sources. At all concentration levels of each combined nitrogen form,  $\overline{\text{RGR}}$  values declined with culture duration. The  $\overline{\text{RGR}}$  values of plants supplied with  $1 \text{ mmol}_\lambda$  of either  $\text{NO}_3^-$ -N,  $\text{NO}_2^-$ -N or  $\text{NH}_2\text{CONH}_2$  were very similar and, after 10 days, were approximately 20 % lower than those recorded for the control plants supplied with atmospheric  $\text{N}_2$ . After 90 days, these  $\overline{\text{RGR}}$  values had decreased to approximately 67 % of the control plants. The  $\overline{\text{RGR}}$  of plants grown on  $1 \text{ mmol}_\lambda \text{NH}_4^-$ -N decreased to 19 % of the control plants after 90 days.

After 15 days growth on different nitrogen sources, the activities of the major nitrogen-assimilating enzymes were measured (Table 53). Nitrogenase activity was highest ( $3105 \text{ nmol} \cdot (\text{g.f.wt})^{-1} \cdot \text{hr}^{-1}$ ) in the control plants provided only with  $\text{N}_2$  and almost zero ( $5 \text{ nmol} \cdot (\text{g.f.wt})^{-1} \cdot \text{hr}^{-1}$ ) in the plants grown in the complete absence of nitrogen. The similar levels

TABLE 52 - The effects of different nitrogen sources on the  $\overline{\text{RGR}}$  ( $\text{g.g}^{-1}.\text{day}^{-1}$ ) of the intact *Azolla filiculoides*-*Anabaena azollae* symbiosis. [Each value is given  $\pm$  standard deviation;  $n = 8$  for all treatments]

Nitrogen source	Conc $\text{t}^-$ (mmol $\text{L}^{-1}$ )	Duration of culture (days)		
		10	30	90
None*	--	0,018 $\pm$ 0,003	0	0
N <sub>2</sub> **	--	0,297 $\pm$ 0,041	0,278 $\pm$ 0,049	0,262 $\pm$ 0,028
NO <sub>3</sub> <sup>-</sup> N*	0,01	0,154 $\pm$ 0,018	0,098 $\pm$ 0,011	0,076 $\pm$ 0,012
	0,1	0,187 $\pm$ 0,029	0,152 $\pm$ 0,021	0,117 $\pm$ 0,015
	1,0	0,241 $\pm$ 0,056	0,214 $\pm$ 0,038	0,175 $\pm$ 0,019
	10,0	0,169 $\pm$ 0,047	0,122 $\pm$ 0,029	0,063 $\pm$ 0,009
NO <sub>2</sub> N*	0,01	0,159 $\pm$ 0,019	0,116 $\pm$ 0,013	0,084 $\pm$ 0,011
	0,1	0,194 $\pm$ 0,037	0,171 $\pm$ 0,030	0,102 $\pm$ 0,017
	1,0	0,231 $\pm$ 0,044	0,209 $\pm$ 0,036	0,170 $\pm$ 0,024
	10,0	0,178 $\pm$ 0,032	0,125 $\pm$ 0,025	0,074 $\pm$ 0,009*
NH <sub>4</sub> <sup>-</sup> N*	0,01	0,150 $\pm$ 0,025	0,093 $\pm$ 0,012	0,042 $\pm$ 0,007
	0,1	0,176 $\pm$ 0,014	0,112 $\pm$ 0,015	0,063 $\pm$ 0,007
	1,0	0,189 $\pm$ 0,029	0,141 $\pm$ 0,020	0,049 $\pm$ 0,005
	10,0	0,138 $\pm$ 0,034	0,042 $\pm$ 0,007	0
NH <sub>2</sub> CONH <sub>2</sub> *	0,01	0,159 $\pm$ 0,026	0,108 $\pm$ 0,016	0,080 $\pm$ 0,010
	0,1	0,199 $\pm$ 0,030	0,161 $\pm$ 0,025	0,122 $\pm$ 0,018
	1,0	0,238 $\pm$ 0,061	0,220 $\pm$ 0,047	0,185 $\pm$ 0,027
	10,0	0,176 $\pm$ 0,052	0,130 $\pm$ 0,029	0,072 $\pm$ 0,013

[\*79,9 % AR: 20 % O<sub>2</sub>: 0,1 % CO<sub>2</sub> in gas phase;

\*\*79,9 % N<sub>2</sub>: 20 % O<sub>2</sub>: 0,1 % CO<sub>2</sub> in gas phase]

TABLE 53 - Comparison of growth rate ( $\overline{\text{RGR}}$ :  $\text{g.g.}^{-1}.\text{day}^{-1}$ ), nitrogenase activity (EPR:  $\text{nmoles.}(\text{g.f.wt})^{-1}.\text{hr}^{-1}$ ), nitrate reductase activity (NaRA:  $\text{nmoles NO}_2\text{-N produced.}(\text{g.f.wt})^{-1}.\text{hr}^{-1}$ ), nitrite reductase activity (NiRA:  $\text{nmoles NO}_2\text{-N used.}(\text{g.f.wt})^{-1}.\text{hr}^{-1}$ ) and nitrogen content (% N) of intact *Azolla filiculoides*-*Anabaena azollae* symbiosis after 15 days growth on different nitrogen sources. [Combined nitrogen supplied at 1 mmol.; each value is given  $\pm$  standard deviation; n = 8 for all treatments]

N Source	$\overline{\text{RGR}}$	EPR	NaRA	NiRA	% N
None	0,009 $\pm$ 0,002 a	5 $\pm$ 1 a	0	80 $\pm$ 20 a	2,01
N <sub>2</sub>	0,285 $\pm$ 0,037 d	3105 $\pm$ 195 d	20 $\pm$ 10 a	390 $\pm$ 50 c	4,95
NO <sub>3</sub> -N	0,226 $\pm$ 0,030 c	2168 $\pm$ 150 c	450 $\pm$ 70 b	420 $\pm$ 70 c	4,15
NO <sub>2</sub> -N	0,220 $\pm$ 0,031 c	2045 $\pm$ 187 c	10 $\pm$ 10 a	430 $\pm$ 60 c	4,08
NH <sub>4</sub> -N	0,164 $\pm$ 0,023 b	85 $\pm$ 16 b	0	220 $\pm$ 40 b	5,69
NH <sub>2</sub> CONH <sub>2</sub>	0,228 $\pm$ 0,024 c	2190 $\pm$ 142 c	30 $\pm$ 20 a	310 $\pm$ 30 c	5,07

[Means within columns followed by similar letters are not significantly different at P = 0,05]

of nitrogenase activity in the plants provided with either NO<sub>3</sub>-N, NO<sub>2</sub>-N or NH<sub>2</sub>CONH<sub>2</sub> were approximately 68 % of the control plants whereas NH<sub>4</sub>-N resulted in very much lower values of nitrogenase activity (3 % of control plants). The highest nitrate reductase activity (450 nmol.(g.f.wt)<sup>-1</sup>.hr<sup>-1</sup>) was measured in those plants provided with NO<sub>3</sub>-N, while very low rates (< 40 nmol.(g.f.wt)<sup>-1</sup>.hr<sup>-1</sup>) were found in plants grown with either atmospheric N<sub>2</sub>, NO<sub>2</sub>-N or NH<sub>2</sub>COHN<sub>2</sub>. No measurable nitrate reductase activity could be detected in plants grown with NH<sub>4</sub>-N or in the complete absence of nitrogen.

Plants supplied with either N<sub>2</sub>, NO<sub>3</sub>-N, NO<sub>2</sub>-N or NH<sub>2</sub>CONH<sub>2</sub> had similar high rates of nitrite reductase activity (> 300 nmol.(g.f.wt)<sup>-1</sup>.hr<sup>-1</sup>). Growth on NH<sub>4</sub>-N caused a 48 % reduction in activity compared to the control plants grown on N<sub>2</sub> while a complete lack of nitrogen in the medium caused an 80 % decrease in activity.

Those plants grown with NH<sub>4</sub>-N as the sole nitrogen source had the highest nitrogen content (5,69 %). Plants supplied with either N<sub>2</sub> or NH<sub>2</sub>CONH<sub>2</sub>

contained approximately 17 % less nitrogen. The nitrogen content of the plants grown on either  $\text{NO}_3\text{-N}$  or  $\text{NO}_2\text{-N}$  was 72 % of those plants provided with  $\text{NH}_4\text{-N}$ . In the complete absence of nitrogen, the plant nitrogen content dropped to 2,01 % or 65 % less than the plants grown on  $\text{NH}_4\text{-N}$ .

The results shown in Table 53 clearly indicated that the enzyme nitrate reductase required a source of  $\text{NO}_3\text{-N}$  for induction. The time course for induction of maximum nitrate reductase activity in the intact association was therefore investigated and found to depend on the concentration of  $\text{NO}_3\text{-N}$  in the growth medium (Table 54). Without prior induction, nitrate reductase activity was barely measurable. At the highest  $\text{NO}_3\text{-N}$  concentration used (10,0  $\text{mmol}_\lambda$ ), induction was complete within one day and the level of activity remained stable for at least six days. At a  $\text{NO}_3\text{-N}$  concentration of 1,0  $\text{mmol}_\lambda$ , induction took two days before stabilizing.

TABLE 54 - The effect of varying  $\text{NO}_3\text{-N}$  concentration and induction time on the rate of nitrate reductase activity (nmoles  $\text{NO}_2\text{-N}$  produced.  $(\text{g.f.wt})^{-1}.\text{hr}^{-1}$ ) in the intact *Azolla filiculoides*-*Anabaena azollae* symbiosis. [Values are given  $\pm$  standard deviation; n = 8 for all treatments]

Nitrate concentration ( $\text{mmol}_\lambda$ )	Time (days) from initiation						
	0	1	2	3	4	5	6
0,01	20 $\pm$ 10	110 $\pm$ 30	170 $\pm$ 20	220 $\pm$ 30	210 $\pm$ 20	240 $\pm$ 30	230 $\pm$ 30
0,1	10 $\pm$ 10	150 $\pm$ 30	250 $\pm$ 30	320 $\pm$ 20	340 $\pm$ 50	350 $\pm$ 30	380 $\pm$ 40
1,0	20 $\pm$ 20	230 $\pm$ 30	400 $\pm$ 30	420 $\pm$ 50	460 $\pm$ 40	430 $\pm$ 40	450 $\pm$ 50
10,0	20 $\pm$ 20	450 $\pm$ 50	420 $\pm$ 40	470 $\pm$ 50	400 $\pm$ 40	410 $\pm$ 50	440 $\pm$ 40

After four days growth on 0,1  $\text{mmol}_\lambda$   $\text{NO}_3\text{-N}$ , nitrate reductase activity reached 79 % of the average maximum rate ( $430\text{nmoles}.\text{(g.f.wt)}^{-1}.\text{hr}^{-1}$ ) attained with higher ( $> 1 \text{ mmol}_\lambda$ )  $\text{NO}_3\text{-N}$  concentrations. Induction of nitrate reductase activity was still incomplete after six days when the *Azolla* plants were grown on 0,01  $\text{mmol}_\lambda$   $\text{NO}_3\text{-N}$ . Therefore, to facilitate the measurement of nitrate reductase activity in all further experiments, without providing unnaturally high concentrations of combined nitrogen, the enzyme was induced by supplying 1,0  $\text{mmol}_\lambda$   $\text{NO}_3\text{-N}$  in the medium 2 days prior

to measurement. This procedure permitted adequate induction of the enzyme without disturbing existing  $\overline{\text{RGR}}$  values or causing drastic changes to the composition of the nutrient medium for prolonged periods.

8.4.2 Location of the major nitrogen assimilating enzymes within the *Azolla filiculoides*-*Anabaena azollae* association

In the *Azolla filiculoides*-*Anabaena azollae* symbiosis, nitrogenase activity was confined to the algal symbiont (Table 55). Alga-free fronds could not reduce acetylene. However, both nitrate and nitrite reductase activity were confined to the fern and no *in vivo* nitrate or nitrite reductase activity could be detected in the algal symbiont (Table 55). Within the fern, approximately 16 % of both nitrate and nitrite reductase activity was located in the roots and the balance (84 %) was located within the fern fronds.

TABLE 55 - Location of nitrogenase activity (EPR: nmoles  $\text{C}_2\text{H}_4$  produced. (g.f.wt)<sup>-1</sup>.hr<sup>-1</sup>), nitrate reductase activity (NaRA: nmoles  $\text{NO}_2\text{-N}$  produced. (g.f.wt)<sup>-1</sup>.hr<sup>-1</sup>) and nitrite reductase activity (NiRA: nmoles  $\text{NO}_2\text{-N}$  used. (g.f.wt)<sup>-1</sup>.hr<sup>-1</sup>) in the *Azolla filiculoides*-*Anabaena azollae* symbiosis. [Nitrate reductase activity induced by two days growth with 1 mmol  $\text{NO}_3\text{-N}$  in medium; Each value is given + standard deviation; n = 4 for all treatments]

Plant portion	EPR	NaRA	NiRA
Intact symbiosis	3090 + 204 a	460 + 50 b	410 + 50 b
Fronds only (with alga) (minus roots)	3415 + 250 a	510 + 60 b	470 + 40 b
Roots only	0	100 + 20 a	90 + 20 a
Alga-free fronds	0	540 + 50 b	500 + 50 b
Freshly isolated <i>A. azollae</i>	29880 + 625 c	0	0
2 month culture of <i>A. azollae</i>	18430 + 440 b	0	0

[Means within columns followed by similar letters are not significantly different at P = 0,05]

The distribution of the major nitrogen-assimilating enzymes within previously-induced symbiotic *A. filiculoides* fronds was investigated with the use of sequential segments of main stem axes. Initial attempts

to use sequentially isolated leaves excised from the main stem axis yielded highly erratic results, possibly due to leaves damaged by the dissection technique. This method was therefore discarded and leaf-bearing stem segments used instead.

Lateral branches were removed first and then the main axis dissected into a series of segments from the stem apex through to the older leaves. The first segment consisted of the stem apex plus leaf primordia and the first two fully-developed leaves. Each subsequent segment consisted of a stem portion bearing four leaves. Since each segment consisted of a very small piece of plant tissue, approximately 500 series of sequential segments had to be dissected in order to obtain sufficient material for each series of enzyme assays. This necessitated an unavoidable delay of up to 24 hours between the time of dissection and the start of a series of enzyme assays.

Each of the three enzymes investigated showed a clear profile of enzyme activity along the stem axis (Table 56). In addition, the percentage dry matter and nitrogen content decreased while the algal heterocyst frequency increased in progressively older segments. Very low nitrogenase activity values were measured in the apical segment ( $335 \text{ nmol. (g.f.wt)}^{-1} \cdot \text{hr}^{-1}$ ). Nitrogenase activity increased to a maximum ( $2240 \text{ nmol. (g.f.wt)}^{-1} \cdot \text{hr}^{-1}$ ) in the third segment (leaves 7 to 10) before declining in subsequent segments. The activity patterns of nitrate and nitrite reductase along the main stem axis were identical. Again, the activity of each enzyme was barely measurable in the apical segment but increased to a maximum ( $> 500 \text{ nmoles. (g.f.wt)}^{-1} \cdot \text{hr}^{-1}$ ) in the fourth and fifth segments (leaves 11 to 18) before declining in the older segments.

The heterocyst frequency of symbiotic *Anabaena azollae* increased progressively from 4,1 % in the apical segment to 31,8 % by the fifth segment, and remained approximately constant in subsequent segments. The dry matter content decreased progressively between the apical and fourth segments before remaining constant (4,5 to 4,7 % N) in older segments. On a dry weight basis, the nitrogen content showed a similar pattern of decrease between the apical and fourth segments followed by a relatively uniform nitrogen content (approximately 3,7 %) in the older segments.

TABLE 56 - Comparison of nitrogenase activity (EPR: nmoles C<sub>2</sub>H<sub>4</sub> produced. (g.f.wt)<sup>-1</sup>.hr<sup>-1</sup>), nitrate reductase activity (NaRA: nmoles NO<sub>2</sub>-N produced. (g.f.wt)<sup>-1</sup>.hr<sup>-1</sup>) and nitrite reductase activity (NiRA: nmoles NO<sub>2</sub>-N used. (g.f.wt)<sup>-1</sup>.hr<sup>-1</sup>) with algal heterocyst frequencies, percentage dry matter and nitrogen content of sequential leaf-bearing segments of *A. filiculoides* main stem axes. [Each value is given ± standard deviation; n = 4 for all treatments; dry matter and nitrogen content values are from pooled samples]

Segment	EPR	NaRA	NiRA	Heterocyst frequency	Percentage dry matter	% N
1: Apex + lvs 1-2	335 ± 42	30 ± 10	20 ± 10	4,1	11,6	6,51
2: lvs 3-6	2160 ± 195	220 ± 30	200 ± 20	14,7	10,2	5,67
3: lvs 7-10	2240 ± 205	390 ± 40	370 ± 50	22,2	5,8	4,85
4: lvs 11-14	1585 ± 127	560 ± 80	530 ± 40	28,5	4,7	3,61
5: lvs 15-18	910 ± 86	550 ± 70	550 ± 60	31,8	4,5	3,68
6: lvs 19-22	520 ± 70	410 ± 50	400 ± 40	32,6	4,7	3,72
7: lvs 23-26	205 ± 22	220 ± 30	200 ± 30	32,5	4,6	3,70

Isolated *A. azollae* was still able to fix atmospheric nitrogen when grown with appreciable quantities of NH<sub>4</sub>-N in the nutrient medium (Table 57). Nitrogenase activity remained approximately constant (76 to 85 nmol. (mg chlorophyll)<sup>-1</sup>.min<sup>-1</sup>) with increasing NH<sub>4</sub>-N concentrations up to 5,0 mmol.l<sup>-1</sup>, but at higher NH<sub>4</sub>-N concentrations (> 6 mmol.l<sup>-1</sup>), nitrogenase activity was reduced. Within each treatment, the level of nitrogenase activity remained constant for at least 10 hours.

#### 8.4.3 Environmental regulation of nitrogen assimilation

Since it was not possible to carry out assays for nitrate reductase and nitrite reductase in the field, this part of the study was conducted in the laboratory. The range of each environmental variable investigated was chosen so as to include conditions pertaining in the field. Assays for nitrogenase activity were conducted under both field and laboratory conditions.

TABLE 57 - The effect of  $\text{NH}_4\text{-N}$  concentration on the nitrogenase activity of isolated *A. azollae*. (Photoperiod: 14L/10D; light intensity: 5 kLux; temperature: 20 °C). [Values are given  $\pm$  standard deviation; n = 8 for each treatment]

$\text{NH}_4\text{-N}$ concentration in medium (nmol.l <sup>-1</sup> )	Nitrogenase activity of <i>A. azollae</i> (nmoles. (mg.chlorophyll) <sup>-1</sup> .min <sup>-1</sup> )
0	85 $\pm$ 7
1	81 $\pm$ 6
3	82 $\pm$ 8
5	76 $\pm$ 5
7	50 $\pm$ 6
9	9 $\pm$ 2

The levels of nitrogenase activity and nitrate and nitrite reductase activity in *A. filiculoides* were found to be dependent on the photoperiod under which the plants had been grown (Table 58). The activity of each enzyme increased when the photoperiod was increased from 6L/18D to 14L/10D. At longer photoperiods there was no significant ( $P > 0,05$ ) increase in enzyme activity.

TABLE 58 - The effect of photoperiod on nitrogenase activity (EPR: nmoles  $\text{C}_2\text{H}_4$  produced. (g.f.wt)<sup>-1</sup>.hr<sup>-1</sup>), nitrate reductase activity (NaRA: nmoles  $\text{NO}_2\text{-N}$  produced. (g.f.wt)<sup>-1</sup>.hr<sup>-1</sup>) and nitrite reductase activity (NiRA: nmoles  $\text{NO}_2\text{-N}$  used. (g.f.wt)<sup>-1</sup>.hr<sup>-1</sup>) in symbiotic *A. filiculoides* plants. [Values are given  $\pm$  standard deviation; n = 5 for all treatments]

Photoperiod	EPR	NaRA	NiRA
6L/18D	945 $\pm$ 43 a	190 $\pm$ 30 a	190 $\pm$ 20 a
10L/14D	1610 $\pm$ 105 b	320 $\pm$ 50 b	300 $\pm$ 30 b
14L/10D	2840 $\pm$ 142 c	550 $\pm$ 80 c	510 $\pm$ 70 c
18L/6D	2975 $\pm$ 125 c	610 $\pm$ 70 c	580 $\pm$ 80 c

[Means within columns followed by the same letter are not significantly different at  $P = 0,05$ ]

All three enzymes showed increased activity as light intensity increased from 1 kLux to 30 kLux (Table 59). At light intensities above 30 kLux the enzyme activity showed a slight, but not significant ( $P > 0,05$ ) decrease.

TABLE 59 - The effect of light intensity on nitrogenase activity (EPR: nmoles  $C_2H_4$  produced. (g.f.wt) $^{-1}$ .hr $^{-1}$ ), nitrate reductase activity (NaRA: nmoles  $NO_2-N$  produced. (g.f.wt) $^{-1}$ .hr $^{-1}$ ) and nitrite reductase activity (NiRA: nmoles  $NO_2-N$  used. (g.f.wt) $^{-1}$ .hr $^{-1}$ ) in symbiotic *A. filiculoides* plants. (Photoperiod: 14L/10D). [Values are given  $\pm$  standard deviation; n = 4 for all treatments]

Light intensity (kLux)	EPR	NaRA	NiRA
1	120 $\pm$ 36 a	140 $\pm$ 20 a	110 $\pm$ 30 a
5	615 $\pm$ 84 b	290 $\pm$ 40 b	280 $\pm$ 40 b
15	1540 $\pm$ 141 c	430 $\pm$ 30 c	430 $\pm$ 40 c
30	2955 $\pm$ 187 d	640 $\pm$ 70 d	620 $\pm$ 60 d
45	2910 $\pm$ 152 d	630 $\pm$ 50 d	620 $\pm$ 80 d
60	2885 $\pm$ 195 d	570 $\pm$ 70 d	570 $\pm$ 50 d

[Means within columns followed by the same letter are not significantly different at  $P = 0,05$ ]

Changes in light quality caused marked changes in *A. filiculoides* nitrogenase activity (Table 60). In green light, nitrogenase activity was significantly higher ( $P < 0,05$ ) than values under either red or blue light, but only 28 % of the control (white light) plants. However, the three- to four-fold drop in light intensity, due to the density of the green filter, could have accounted for the low nitrogenase activity recorded under green light. Nitrate and nitrite reductase activity under blue, green and red light did not differ significantly ( $P > 0,05$ ) from each other, but were significantly lower ( $P < 0,05$ ) than the corresponding activity recorded under white light.

TABLE 60 - The effect of light quality on nitrogenase activity (EPR: nmoles C<sub>2</sub>H<sub>4</sub> produced.(g.f.wt)<sup>-1</sup>.hr<sup>-1</sup>), nitrate reductase activity (NaRA: nmoles NO<sub>2</sub>-N produced.(g.f.wt)<sup>-1</sup>.hr<sup>-1</sup>) and nitrite reductase activity (NiRA: nmoles NO<sub>2</sub>-N used.(g.f.wt)<sup>-1</sup>.hr<sup>-1</sup>) in symbiotic *A. filiculoides* plants. (Photoperiod: 14L/10D; Light intensity: 30 kLux). [Values are given ± standard deviation; n = 4 for all treatments]

Filter transmission maxima (nm)	EPR	NaRA	NiRA
Blue (450)	20 <u>±</u> 8 a	190 <u>±</u> 50 a	190 <u>±</u> 30 a
Green (530)	710 <u>±</u> 68 b	290 <u>±</u> 50 a	280 <u>±</u> 60 a
Red (655)	35 <u>±</u> 9 a	220 <u>±</u> 40 a	220 <u>±</u> 40 a
White (400-700)	2450 <u>±</u> 184 c	490 <u>±</u> 70 b	480 <u>±</u> 80 b

[Means within columns followed by the same letters are not significantly different at P = 0,05]

Each of the three enzymes studied showed similar patterns of *in vivo* activity in response to changes in culture pH (Table 61). In the pH range 5 to 8, the activity of each enzyme did not change significantly (P > 0,05). However, all enzymes had significantly (P < 0,05) lower activity at both pH 4 and pH 9.

The effect of constant temperature on *in vivo* rates of nitrogen assimilation by symbiotic *A. filiculoides* plants was investigated over the temperature range 5 to 40 °C (Table 62). With increasing temperature, nitrogenase activity increased to a maximum (3090 nmol.(g.f.wt)<sup>-1</sup>.hr<sup>-1</sup>) at approximately 25 °C but decreased at higher temperatures with no activity occurring at 40 °C. Nitrate and nitrite reductase activities followed similar trends, increasing to a maximum between 25 and 30 °C, and then declining at higher temperatures. At both 5 °C and 40 °C nitrate and nitrite reductase activities were barely measurable.

TABLE 61 - The effect of pH on nitrogenase activity (EPR: nmoles C<sub>2</sub>H<sub>4</sub> produced. (g.f.wt)<sup>-1</sup>.hr<sup>-1</sup>), nitrate reductase activity (NaRA: nmoles NO<sub>2</sub>-N produced. (g.f.wt)<sup>-1</sup>.hr<sup>-1</sup>) and nitrite reductase activity (NiRA: nmoles NO<sub>2</sub>-N used. (g.f.wt)<sup>-1</sup>.hr<sup>-1</sup>) in symbiotic *A. filiculoides* plants. (Photoperiod: 14L/10D; Light intensity: 30 kLux). [Values are given + standard deviation; n = 4 for all treatments]

pH	EPR	NaRA	NiRA
4	1545 + 142 a	270 + 30 a	270 + 30 a
5	2860 + 148 b	520 + 50 b	500 + 40 b
6	3010 + 180 b	600 + 70 b	590 + 60 b
7	2785 + 155 b	550 + 60 b	550 + 70 b
8	2960 + 167 b	540 + 60 b	530 + 40 b
9	1380 + 128 a	200 + 40 a	190 + 20 a

[Means within columns followed by the same letter do not differ significantly at P = 0,05]

TABLE 62 - The effect of temperature on nitrogenase activity (EPR: nmoles C<sub>2</sub>H<sub>4</sub> produced. (g.f.wt)<sup>-1</sup>.hr<sup>-1</sup>), nitrate reductase activity (NaRA: nmoles NO<sub>2</sub>-N produced. (g.f.wt)<sup>-1</sup>.hr<sup>-1</sup>) and nitrite reductase activity (NiRA: nmoles NO<sub>2</sub>-N used. (g.f.wt)<sup>-1</sup>.hr<sup>-1</sup>) in symbiotic *A. filiculoides* plants. [values are given + standard deviation; n = 4 for all treatments]

Temperature	EPR	NaRA	NiRA
5	430 + 45 a	30 + 10 a	20 + 10 a
10	710 + 82 a	90 + 20 a	80 + 20 a
15	1625 + 134 b	190 + 30 b	170 + 30 b
20	2750 + 215 c	280 + 40 b	270 + 30 b
25	3090 + 250 c	480 + 50 c	480 + 40 c
30	2180 + 198 b	530 + 50 c	510 + 50 c
35	325 + 92 a	300 + 30 b	290 + 20 b
40	0	20 + 10 a	20 + 10 a

[Means within columns followed by the same letter are not significantly different at P = 0,05]

When compared with the enzyme activity measured under constant temperatures, a 10 °C temperature differential between diurnal and nocturnal temperatures permitted *A. filiculoides* plants to assimilate nitrogen at higher temperatures (Table 63). Nitrogenase activity increased with increasing temperatures to a maximum between 25/15 °C and 30/20 °C. Despite a decrease in the rate of nitrogenase activity at 40/30 °C, measurable rates were still recorded. Nitrate and nitrite reductase activity was maximal between 30/20 °C and 35/25 °C, but at higher temperatures, activity was decreased. Both enzymes had very low levels of activity in the lowest temperature regime used (10/5 °C).

TABLE 63 - The effect of different diel temperature regimes on nitrogenase activity (EPR: nmoles C<sub>2</sub>H<sub>4</sub> produced. (g.f.wt)<sup>-1</sup>.hr<sup>-1</sup>), nitrate reductase activity (NaRA: nmoles NO<sub>2</sub>-N produced. (g.f.wt)<sup>-1</sup>.hr<sup>-1</sup>) and nitrite reductase activity (NiRA: nmoles NO<sub>2</sub>-N used. (g.f.wt)<sup>-1</sup>.hr<sup>-1</sup>) in symbiotic *A. filiculoides* plants. (Photoperiod: 14L/10D; light intensity: 30 kLux). [Values are given ± standard deviation; n = 4 for all treatments]

Temp. (°C) Day/Night	EPR	NaRA	NiRA
10/5	245 ± 60 a	40 ± 20 a	20 ± 10 a
15/5	850 ± 72 a	110 ± 20 a	100 ± 20 a
20/10	1885 ± 136 b	260 ± 40 b	250 ± 40 b
25/15	2820 ± 215 c	340 ± 50 b	340 ± 50 b
30/20	2980 ± 240 c	500 ± 60 c	490 ± 50 c
35/25	1755 ± 140 b	480 ± 50 c	480 ± 60 c
40/30	85 ± 47 a	140 ± 20 a	130 ± 20 a

[Means within columns followed by the same letter are not significantly different at P = 0,05]

Different degrees of osmotic stress, achieved by the addition of polyethylene glycol (PEG-6000) to the basal growth medium, caused marked changes in rates of nitrogen assimilation (Table 64). Nitrogenase activity dropped to zero at an osmotic stress of -5 bars. In contrast, the activities of both nitrate and nitrite reductase remained constant at osmotic stresses up to -5 bars. However, further increases in osmotic stress to -9 bars

caused a 60 % reduction in the activity of both enzymes below the control (no extra osmotic stress) treatment.

TABLE 64 - The effect of increasing osmotic stress ( $\psi_s$ ) on nitrogenase activity (EPR: nmoles  $C_2H_4$  produced.(g.f.wt) $^{-1}$ .hr $^{-1}$ ), nitrate reductase activity (NaRA: nmoles  $NO_2-N$  produced.(g.f.wt) $^{-1}$ .hr $^{-1}$ ) and nitrite reductase activity (NiRA: nmoles  $NO_2-N$  used.(g.f.wt) $^{-1}$ .hr $^{-1}$ ) in symbiotic *A. filiculoides* plants. (Photoperiod: 14L/10D; light intensity: 30 kLux; temperature: 25 °C). [Values are given  $\pm$  standard deviation; n = 4 for each treatment]

$\psi_s$ (bars)	EPR	NaRA	NiRA
0	2855 $\pm$ 245 c	540 $\pm$ 50 c	520 $\pm$ 60 c
- 1	1780 $\pm$ 142 b	560 $\pm$ 60 c	530 $\pm$ 40 c
- 3	425 $\pm$ 38 a	530 $\pm$ 40 c	530 $\pm$ 60 c
- 5	0	580 $\pm$ 70 c	550 $\pm$ 70 c
- 7	0	390 $\pm$ 30 b	380 $\pm$ 30 b
- 9	0	270 $\pm$ 20 a	210 $\pm$ 20 a

[Means within columns followed by the same letter are not significantly different at P = 0,05]

In laboratory trials, the process of mat formation had pronounced effects on rates of nitrogen assimilation (Table 65). In this experiment, *A. filiculoides* plants were grown in asbestos trays (0,21 m<sup>2</sup> surface area) on N-free Azolla-I medium. Before crowding became evident (at approximately day 20), nitrogenase activity was high (> 2100 nmol.(g.f.wt) $^{-1}$ .hr $^{-1}$ ) and nitrate reductase activity was very low (< 60nmol.(g.f.wt) $^{-1}$ .hr $^{-1}$ ). As the plants became crowded together, a multi-layered mat formed, and nitrogenase activity decreased while nitrate reductase activity rose to a maximum (520nmol.(g.f.wt) $^{-1}$ .hr $^{-1}$ ).

Regular chemical analysis of the underlying nutrient solution showed that once a multi-layered mat had formed (at approximately day 25), increasing quantities of combined nitrogen were found in the nutrient medium. From day 25 when the first traces of combined nitrogen were found the concentration increased steadily to 0,93 mg.l $^{-1}$   $NH_4-N$  and 0,58 mg.l $^{-1}$

NO<sub>3</sub>-N on day 50. In addition, a few senescent plants became waterlogged and sank to the bottom of the tray while several plants shed some of their roots into the nutrient medium. These particulate forms of nitrogen contributed, on average, 9,34 mg nitrogen per tray (0,89 mg.ℓ<sup>-1</sup>). On average, the medium in each tray contained 25,5 mg of nitrogen at the end of the experiment (2,43 mg.ℓ<sup>-1</sup>), 36 % in particulate forms and 62 % as a mixture of NO<sub>3</sub>-N and NH<sub>4</sub>-N. This quantity of nitrogen in the medium (25,5 mg) amounted to approximately 2 % of the nitrogen fixed by *A. filiculoides* during the fifty-day experiment.

TABLE 65 - The changes in nitrogenase activity (EPR: nmoles C<sub>2</sub>H<sub>4</sub> produced.(g.f.wt)<sup>-1</sup>.hr<sup>-1</sup>) and nitrate reductase activity (NaRA: nmoles NO<sub>2</sub>-N produced.(g.f.wt)<sup>-1</sup>.hr<sup>-1</sup>) with time during mat formation in the laboratory. (Plants grown at 25 °C, pH 7,0, and 14L/10D photoperiod). [Values are given ± standard deviation; n = 3 for all treatments]

Time (days)	EPR	NaRA
0	2895 ± 230 c	20 ± 10 a
10	2910 ± 215 c	30 ± 20 a
20	2100 ± 220 c	50 ± 20 a
30	925 ± 130 b	220 ± 30 b
40	400 ± 88 a	490 ± 40 c
50	165 ± 26 a	520 ± 50 c

[Means within columns followed by the same letters are not significantly different at P = 0,05]

### 8.5 DISCUSSION

While several investigators have studied nitrogen fixation in different species of *Azolla*, the only quantitative data on the *Azolla* nitrate-nitrite reductase system has been provided by Holst and Yopp (1979b, c) for *A. mexicana*. The effects of different environmental variables on nitrogenase activity in symbiotic *A. filiculoides* plants have been described in detail in Sections 5.3 and 5.4 of this dissertation. However, in order to investigate environmental regulation of both nitrogen assimilation pathways, several of these experiments had to be repeated to

permit simultaneous comparisons of nitrogenase activity and the activity of the nitrate-nitrite reductase system.

The ability of symbiotic *A. filiculoides* plants to grow on either nutrient media containing combined nitrogen in the absence of atmospheric  $N_2$  or nitrogen-free media in the presence of atmospheric  $N_2$  clearly demonstrates the presence of two pathways of nitrogen assimilation. Sustained growth under these conditions indicates that both of these assimilatory pathways can be utilized independently to fulfil the total nitrogen requirements of the association. This is in accord with earlier workers (Peters and Mayne, 1974b; Peters, 1975, 1976) who reported that *A. caroliniana* could sustain growth either via nitrogen fixation or by the uptake of combined nitrogen.

During this investigation, *A. filiculoides* registered highest  $\overline{RGR}$  values when provided with atmospheric  $N_2$ , confirming earlier reports for *A. caroliniana* by Peters *et al.* 1976, 1977) and Peters (1977). However, the  $\overline{RGR}$  values of plants provided with a source of combined nitrogen were always lower and depended both on the concentration and the ionic form of the nitrogen supplied. *Azolla filiculoides* plants provided with  $NH_4-N$  regressed rapidly while the provision of either  $NO_3-N$ ,  $NO_2-N$  or  $NH_2CONH_2$  maintained  $\overline{RGR}$  values at approximately 80 % of the atmospheric  $N_2$ -grown plants.

Since absorbed nitrogen can only be incorporated into organic forms when it is in the reduced form,  $NH_4-N$  (Haynes and Goh, 1978), the ability of *A. filiculoides* to sustain growth on  $NO_3-N$  implies the existence of the enzymes nitrate reductase and nitrite reductase. These enzymes are necessary for the reduction of  $NO_3-N$  to  $NH_4-N$  and have been found in numerous plant genera (Haynes and Goh, 1978). *In vivo* assays (Klepper *et al.*, 1971) have demonstrated the presence of both nitrate reductase and nitrite reductase in symbiotic *A. filiculoides* plants, supporting the observations of Holst and Yopp (1979b, c) on *A. mexicana*. In this study on *A. filiculoides*, both enzymes were shown to have similar levels of activity. In time-course enzyme assays, the activity of each enzyme increased linearly, without an initial lag phase, from initiation of the assay.

In the intact *A. filiculoides* symbiosis, nitrogenase activity is diminished in those plants provided with a source of combined nitrogen. Since the ultimate product of both nitrogen fixation and the nitrate-nitrite reductase system is ammonia (Haynes and Goh, 1978), the lower nitrogenase activity in nitrate-grown plants could have been due to feedback inhibition as suggested by Peters and Mayne (1974b).

Within the *Azolla filiculoides*-*Anabaena azollae* symbiosis, nitrogenase activity is confined to the algal symbiont. Alga-free fronds do not fix atmospheric nitrogen. The observations by Walmsley *et al.* (1973) and Peters and Mayne (1974b) that alga-free *Azolla* plants can only be maintained in culture when supplied with a source of combined nitrogen have been confirmed in this study. Freshly isolated *Anabaena azollae* material has high levels of nitrogenase activity which become diminished by up to 35 % with prolonged culture on N-free media. The levels of nitrogenase activity reported by Newton and Herman (1979) for *A. azollae* isolated from *A. caroliniana* are similar to those of free-living *Anabaena flos-aquae* (Neilson, Rippka and Kunisawa, 1971) but only 20 to 50 % of the levels recorded in this study. This discrepancy is most probably due to differences in culture conditions.

Examination of the levels of enzyme activity in *A. filiculoides* plants grown on different nitrogen sources indicates that the nitrite reductase activity is constitutive while nitrate reductase requires a supply of  $\text{NO}_3\text{-N}$  for induction. This supported the findings of Beevers and Hageman (1969) who indicated that nitrate reductase activity declines in the absence of  $\text{NO}_3\text{-N}$  because of a continual need for substrate induction. Holst and Yopp (1979c) reported that maximum nitrate reductase activity in *A. mexicana* requires 5 mmol.  $\text{NO}_3\text{-N}$  for induction and that the levels of enzyme activity stabilize after 24 hours of induction. Smith and Thompson (1971) reported similar  $\text{NO}_3\text{-N}$  requirements for nitrate reductase induction in excised barley shoots. However, these concentrations of  $\text{NO}_3\text{-N}$  are unnaturally high when compared to field conditions. In this investigation, a time-course study revealed that adequate induction of nitrate reductase occurs within two days when *A. filiculoides* plants are supplied with 1 mmol.  $\text{NO}_3\text{-N}$ .

The enzymes nitrate reductase and nitrite reductase appear to be confined to the fern component since neither enzyme could be detected within the algal symbiont. Holst and Yopp (1979c) found the identical situation in *A. mexicana*, and reported that 14 % of the total nitrate reductase activity is located in the fern's roots. This is in agreement with the present study, where 16 % of the activity of both nitrate reductase and nitrite reductase was found to be located within *A. filiculoides* roots while the balance (84 %) of each enzyme was located within the fern's fronds. This is consistent with the alga-free fern's ability to utilize  $\text{NO}_3\text{-N}$  as a source of combined nitrogen. However, the presence of nitrate reductase within both roots and fronds indicates that a small proportion of the absorbed  $\text{NO}_3\text{-N}$  is reduced to  $\text{NH}_4\text{-N}$  before being transported to the fronds while a larger proportion of the  $\text{NO}_3\text{-N}$  is first transported to the fronds before being reduced to  $\text{NH}_4\text{-N}$ . Thus, the contention by Peters (1977) that the algal symbiont may not in fact come into direct contact with either  $\text{NO}_3\text{-N}$  or  $\text{NO}_2\text{-N}$  may not be valid. However, evidence to support Peters' (1977) claim is provided by the lack of an inhibitory effect of  $\text{NO}_3\text{-N}$  on *A. azollae* heterocyst frequencies (Hill, 1975), while  $\text{NO}_3\text{-N}$  is reported to cause lower heterocyst frequencies in free-living species of *Anabaena* (Fay, Stewart, Walsby and Fogg, 1968). In addition, the algal symbiont is not in direct contact with either the external environment or the vascular system of the plant (Peters, Ray *et al.*, 1980). Nevertheless, the lack of a "nitrate effect" on *A. azollae* heterocyst frequencies may rather indicate that *A. azollae* is less sensitive to  $\text{NO}_3\text{-N}$  concentrations than free-living species of *Anabaena*.

In the intact *Azolla filiculoides*-*Anabaena azollae* symbiosis used in this study, the algal symbiont was found to undergo a pattern of development and differentiation parallel to that of the fern (Section 4.4.4), confirming the earlier studies of Konar and Kapoor (1972), Hill (1977) and Peters *et al.* (1978) for other *Azolla* species. Algal heterocysts, the sites of nitrogen fixation (Fay *et al.*, 1968), rapidly increase in frequency from the fern apex to senescent leaves. Observations on *A. filiculoides* indicate that nitrogenase-catalyzed ethylene production is low in the apex, increases rapidly as leaves mature and then declines as leaves senesce. These results support the findings of Hill (1977), Becking (1978), Peters, Ray *et al.* (1980) and Kaplan and Peters (1981).

Slight differences in the rapidity of the sequence and rates of ethylene production in these studies can be attributed to differential responses of the *Azolla* species used and their culture conditions.

In agreement with the report by Kaplan and Peters (1981) for *A. caroliniana* the nitrogen content of *A. filiculoides* leaves decreases with increasing leaf age. Thus, the increase in nitrogenase activity and decrease in nitrogen content as a function of leaf age imply the transfer of fixed nitrogen from mature leaves to the shoot apex. Increased levels of cellular nitrogen in blue-green algae inhibit the formation of heterocysts and thereby prevent nitrogen fixation (Kulasooriya, Lang and Fay, 1972). Therefore, the supply of fixed nitrogen to the fern apex could maintain algal growth, but prevent heterocyst formation, whilst depletion of nitrogen in the older leaves could stimulate further heterocyst formation (Kaplan and Peters, 1981). Kaplan and Peters (1981) have further suggested that the simple and branched hairs with transfer cell morphology (Duckett *et al.*, 1975b) that are present in both *Azolla* leaf cavities and stem apices (Calvert and Peters, 1981) might be the pathway via which carbon and nitrogen compounds are transferred between fern and alga.

The observation during this study that isolated *A. azollae* is able to withstand up to 5 mmol.  $\text{NH}_4\text{-N}$  in the medium without appreciable loss of nitrogenase activity, combined with the results of other workers (Newton and Cavins, 1976; Peters, 1977; Peters, Ray *et al.*, 1980) that up to 50 % of the nitrogen fixed by the isolated endophyte is released as  $\text{NH}_4\text{-N}$ , implicates ammonia ( $\text{NH}_4\text{-N}$ ) as the nitrogen compound that is transferred from alga to fern. Evidence to support this hypothesis is provided by Newton and Cavins (1976) who recorded high levels of intracellular (i.e. intra-cavity)  $\text{NH}_4\text{-N}$  in intact *A. caroliniana* plants and Peters *et al.* (1979) who demonstrated that fixed  $^{15}\text{N}_2$  was rapidly assimilated and incorporated into amino acids and protein in the fern tissue. The finding by Ray *et al.* (1979) that virtually all the activity of the major aminating enzymes glutamate dehydrogenase and glutamine synthetase is found in the *Azolla* host also provides further support. These features of the *Azolla-Anabaena* symbiosis conform to the scheme suggested by Stewart (1978) and Haselkorn (1978) where, in plant-cyanophyte associations, the endophyte's glutamine synthetase activity or its synthesis are inhibited by the plant. Therefore, the proposal put forward by Kaplan and Peters (1981), that the

"ammonia released by the endophyte in mature leaf cavities might be metabolized to glutamine by the glutamine synthetase of the host fern and then transported within the fern", can account for the above observations.

In general, the characteristics of the nitrate-nitrite reductase system reported for *A. filiculoides* in this study are similar to those recorded for higher plants (Beevers and Hageman, 1969; Klepper *et al.*, 1971; Haynes and Goh, 1978). However, several interesting differences were noted when the enzymes of the two nitrogen assimilation pathways in the intact *A. filiculoides* symbiosis were compared as to their response to different environmental parameters. For many of the parameters investigated, it has been shown that the fern's nitrate-nitrite reductase system is capable of tolerating a wider range of variation of most parameters than the nitrogenase system of the alga. Particularly important in this regard is the ability of the fern's nitrate-nitrite reductase system to function under higher levels of osmotic stress and temperature. The exceptions to the above appear to be light quality where the algal nitrogenase can utilize green light, and photoperiod and culture pH, where both enzyme systems show identical responses. Thus, despite the protection from certain chemical factors afforded to the alga by the fern, the algal nitrogenase system is more susceptible to most environmental factors than the fern's nitrate-nitrite reductase system. Similar features have been reported for *A. mexicana* by Holst and Yopp (1979b). In most studies of the effects of combined nitrogen on rates of nitrogen assimilation by species of *Azolla* have employed unnaturally high ( $> 50 \text{ mg.l}^{-1}$ ) nitrogen concentrations (Peters and Mayne, 1974b; Peters, 1975; Brotonegoro and Abdulkadir, 1976; Singh, 1977a; Subudhi and Singh, 1979b; Yatazawa *et al.*, 1980). These high concentrations bear little or no relationship to conditions occurring in the field and the applicability of these findings to the conditions affecting natural *Azolla* populations is often tenuous. In most natural aquatic systems, concentrations of combined nitrogen seldom rise above  $1 \text{ mg.l}^{-1}$ , and then usually only under anaerobic or partly anaerobic conditions. As demonstrated by this study, low concentrations of combined nitrogen ( $< 5 \text{ mg.l}^{-1}$ ) can subtly regulate the rates of both nitrogen fixation and nitrate reduction in *A. filiculoides*.

Under optimal laboratory conditions the algal nitrogenase system fixes atmospheric  $N_2$  at a rate 3,8 to 5,0 times faster than the reduction of  $NO_3-N$  to  $NH_4-N$  by the fern's nitrate-nitrite reductase system. However, when *A. filiculoides* plants are grown under sub-optimal conditions, algal nitrogen fixation rates decline rapidly while rates of nitrate reduction remain unchanged resulting in an increase in the relative importance of the fern's nitrate-nitrite reductase system. This feature is of particular importance when considering rates of nitrogen assimilation during mat formation. The laboratory studies showed that at the monolayer stage and before the onset of crowding, the nitrate-nitrite reductase system provides only 0,2 % of the total quantity of nitrogen assimilated per hour. The formation of a multi-layered mat causes a decrease in nitrogenase activity and an increase in the rate of nitrate reduction. Thus, despite a 90 % drop in the total amount of nitrogen assimilated by the symbiosis per hour, the nitrate-nitrite reductase system can provide 24 % of the total nitrogen assimilated in a multi-layered mat. With the formation of a multi-layered *Azolla* mat under both laboratory and field conditions, the lower layers of the mat become senescent and waterlogged and begin to decompose, releasing much of their nitrogen into the water (Section 6.3.7). The presence of this combined nitrogen causes a partial inhibition of nitrogen fixation by the remaining *Azolla* plants and, at the same time, induces increased activity of the nitrate-nitrite reductase system. This enzyme system is then able to assimilate much of the combined nitrogen present in the underlying water to compensate for the loss of fixed atmospheric  $N_2$ .

The possession of two nitrogen assimilation pathways thus confers a considerable competitive advantage to symbiotic *Azolla* plants. Fixation of atmospheric nitrogen by the algal symbiont permits *Azolla* to colonize nitrogen-deficient waters which cannot support the growth of non-nitrogen-fixing aquatic plants. Once established, the formation of multi-layered *Azolla* mats cause drastic changes to the chemistry of the underlying waters, which in turn stimulate the fern component of the symbiosis to assimilate combined nitrogen from the water. Thus, the chemical modifications to the environment under a multilayer mat are advantageous to the *Azolla-Anabaena* association.

## CHAPTER 9

### CONCLUDING DISCUSSION

In this autecological investigation of *Azolla filiculoides*, several aspects of the fern-alga association have been studied. Each of these aspects has had to be considered independently since they were characterized by specific problems related to both methodology and interpretation. Although each section included in this thesis had discrete objectives, the overall aim of the study was to obtain a better understanding of the role played by *A. filiculoides* in the Hendrik Verwoerd Dam catchment area. This chapter is therefore intended as an overall synthesis of the findings obtained during this study.

Identification of the *Azolla* material from the Hendrik Verwoerd Dam catchment area as *A. filiculoides* (Twyman and Ashton, 1972) has provided the first conclusive identification of the species since its introduction into South Africa approximately twenty years previously (Oosthuizen and Walters, 1961). Personal collections and the examination of herbarium records indicated that *A. filiculoides* is confined to temperate highveld regions and that the Hendrik Verwoerd Dam is the focal point of its rather limited distribution in southern Africa. The two species of *Azolla* indigenous to southern Africa, *A. nilotica* and *A. pinnata*, are confined to tropical and sub-tropical regions of the sub-continent.

South African specimens of *A. filiculoides* are morphologically and anatomically identical to published descriptions of European and North American material of this species (Campbell, 1893; Duncan, 1940; Svenson, 1944; Bonnet, 1957). All the *A. filiculoides* material examined in this study contained colonies of the endosymbiotic blue-green alga *Anabaena azollae* within dorsal leaf lobe cavities and no naturally-occurring alga-free plants were found. The algal cells undergo a chronological development parallel to the development of the fern, with compatible rates of cell division to prevent the fern outgrowing the alga and *vice versa*. The mechanisms whereby this developmental sequence is regulated are not yet known.

At the fern apex, all algal cells are generative and no heterocysts are seen. With the development of leaf primordia, algal filaments become trapped in developing cavities in the dorsal leaf lobe and heterocyst differentiation is initiated. Further leaf development results in closure of the leaf cavity except for a small pore to the exterior and an increase in heterocyst frequencies up to approximately 33 %. At all stages of *Azolla* leaf development, the leaf cavity contains both simple and branched hairs. These hairs are characterized by the presence of elaborate cell wall ingrowths and numerous organelles consistent with transfer cell ultrastructure. Peters *et al.* (1978) have also shown that all of these leaf cavity hairs do not in fact have a similar ultrastructure as implied by Duckett *et al.* (1975), but that consistent differences exist between the ultrastructure of hairs from the fern apex and mature leaf cavities.

The size range of *A. azollae* cells from South African *A. filiculoides* material agree with published descriptions of *A. azollae* found in other *Azolla* species (e.g. Tilden, 1910; Shen, 1960; Hill, 1977). Much of the reported variability in *A. azollae* cell sizes can be attributed to the alga's developmental sequence within the fern's leaf cavities. The algal cell dimensions depend on the leaf position (i.e. age) from where the cells are taken, since the algal cells enlarge considerably with increasing age. At the ultrastructural level, the structure of *A. azollae* heterocysts and vegetative cells is typical of many non-gas vacuolate free-living *Anabaena* species and the cytoplasmic inclusions are identical to those found in other blue-green algae. Despite the morphological similarity between *A. azollae* and *A. variabilis* and a recent report that these species show similar fluorescent-antibody staining reactions (Gates, Fisher, Goggin and Azrolan, 1980), it is unlikely that *A. azollae* is merely an ecoform of *A. variabilis* as suggested by Fjerdingsstad (1976). This conclusion is supported by physiological and biochemical evidence obtained in this and earlier studies (Newton and Cavins, 1976; Peters, 1976, 1977; Newton and Herman, 1979) that *A. azollae* is physiologically distinct from the other free-living species of *Anabaena*.

The isolation and culture of both components of the *Azolla*-*Anabaena* symbiosis during this study provide an important step in understanding the

mechanisms of the symbiotic association. The low frequency of successful *A. azollae* isolates is possibly due to the fact that the total *A. azollae* population in an *Azolla* frond contains only a low proportion of undifferentiated cells capable of *in vitro* growth. The failure of several earlier investigators to isolate *A. azollae* successfully could be attributed to bacterial contamination, incorrect culture conditions or the use of mature *Azolla* leaves containing differentiated *A. azollae* cells incapable of *in vitro* growth. Therefore, future attempts to isolate *A. azollae* should perhaps focus on the undifferentiated cells at the fern apical meristem.

Isolated *A. azollae* is capable of both autotrophic and heterotrophic growth, and also fixes atmospheric nitrogen at very high rates. Similar features have been noted for symbiotic blue-green algae isolated from liverworts (Hoare *et al.*, 1971; Stewart and Rogers, 1978). Thus if, as the observations in this study suggest, the nitrogenase activity in *A. azollae* is preferentially heterotrophic, the alga must depend on the fern for supplies of substrate. Algal heterocysts have also been shown to lack photosystem II and the ability to fix carbon dioxide (Haselkorn, 1978). Therefore, the high algal heterocyst frequencies in mature *Azolla* leaves imply a dependence upon fixed carbon compounds from the fern as a source of reductant to maintain the high rates of nitrogen fixation (Peters *et al.*, 1979; Kaplan and Peters, 1981).

The accumulated evidence indicates that atmospheric nitrogen fixed by *A. azollae* is transported to the fern, metabolized to glutamine by the *Azolla* glutamine synthetase and then transported within the fern. The supply of nitrogen to *A. azollae* cells at the fern apex maintains algal growth and prevents heterocyst formation, while the depletion of nitrogen in the older leaves stimulates further heterocyst formation. The only logical route whereby the two-way transfer of carbon and nitrogen compounds between fern and alga can take place is via the intra-cavity hair cells.

In this study the consistent failure of attempts to recombine cultured *A. azollae* and alga-free *A. filiculoides* prevented the fulfilment of Koch's postulates for symbiotic organisms. A large proportion of these unsuccessful attempts could be attributed to incorrect methodology. How-

ever, the recent report of changed surface antigenicity (Gates *et al.*, 1980) in isolated *A. azollae* has indicated that the physiology of *A. azollae* might have been altered during the isolation process. It is also possible that these changes in surface antigenicity might have been due to an alteration in the specific lectin-receptor recognition system that has been shown to exist in the *Azolla-Anabaena* symbiosis (Kobiler *et al.*, 1981). Further research on these aspects is needed.

The growth and development of *A. filiculoides* plants throughout an annual cycle depends on the presence of suitable habitat, usually a standing or gently-flowing body of water. However, the inherent fragility of *A. filiculoides* causes the plants to fragment rapidly when physically disturbed and adequate growth only occurs when the habitat is sufficiently sheltered from excessive wind and wave action. *Azolla filiculoides* plants fragment rapidly and die on exposed areas of open water where wind and wave action is high and a large degree of turbulence exists.

The seasonality of rainfall in the catchment area results in wide fluctuations in the flow of the Orange River and necessitates using much of the Hendrik Verwoerd Dam's large drawdown capacity each year to provide water for irrigation purposes and compensation river-flow. These variations in the reservoir's water level result in periodic inundation and exposure of wide areas of shoreline and prevent the development of a stable marginal macrophyte community. As a result, the extensive wind and wave action causes the formation of a barren, rock-strewn shoreline with no sheltered habitat suitable for the growth of *A. filiculoides*. However, many of the small streams flowing into the Hendrik Verwoerd Dam are narrow (5 to 10 m wide) and often deeply incised, such that their high banks offer a large measure of protection from wind action. Most of these streams are slow-flowing for the greater part of the year (> 90 %), occasionally drying up into a series of long (up to 500 m) pools, and provide ideal sheltered habitat for *A. filiculoides*. Large stretches of these streams can become completely covered with *A. filiculoides* mats for between six and eight months each year until annual summer floods flush out most of the plants into the lake. Surveys of the open waters and shoreline of the Hendrik Verwoerd Dam show that these *A. filiculoides* plants are of little significance to the lake since they are rapidly dis-

persed and fragmented, and soon die. After the floods, those plants which survive in the streams then proliferate to reform the original mats. The flooding therefore gives rise to seasonal fluctuations in the population density of *A. filiculoides* in these streams and the system can be considered to be self-regulatory.

In addition to the requirement for a suitable habitat, the growth of *A. filiculoides* depends on an adequate supply of nutrients and favourable environmental conditions. Analyses of the nutrient content at field sites indicate that nutrient availability is probably the major factor limiting *A. filiculoides* growth in the field.

Symbiotic *A. filiculoides* plants require an adequate supply of all the usual macro- and micronutrients to sustain growth and nitrogen fixation. Deficiencies or excesses of most nutrients are inhibitory. Continuous culture experiments demonstrated that the nutrient supply rate (a function of flow rate) is important, but that a certain minimum nutrient concentration or threshold level is also necessary. *Azolla filiculoides* plants are very susceptible to inadequate supplies of Ca, P and Fe and deficiency symptoms are rapidly manifested.

In the Hendrik Verwoerd Dam catchment area, nutrient availability is regulated by the hydrochemistry of the water which in turn is dependent on the hydrological cycle and the regional geology. All sampling sites in the Hendrik Verwoerd Dam catchment area have similar low nutrient concentrations with virtually identical ionic proportions. High summer river flows contain heavy silt loads with considerable quantities of adsorbed cations. These ions provide an important extra source of nutrients to the *Azolla* plants growing in riverine sites. The concentrations of all dissolved nutrients are very low in open water riverine sites and the field studies have indicated that concentrations of Ca, P and Fe are below the threshold levels required to sustain adequate growth. In addition, the aerobic conditions present in open water areas ensure that almost all of the dissolved Fe is in the ferric ( $\text{Fe}^{+++}$ ) form and, because of its low solubility, virtually unavailable to *Azolla*. Conversely, dissolved nutrient concentrations beneath established *A. filiculoides* mats are much higher, and though Ca, P and Fe levels are only at or fractionally below

threshold levels, they are sufficiently high enough to sustain adequate growth rates. The higher nutrient concentrations beneath the multi-layered mats are due to the rapid decomposition of *A. filiculoides* plants in the lower mat layers. Particularly important in this regard is the rapid release of P and Fe from the decomposing plants. The development of anaerobic conditions in the water beneath a multi-layered *A. filiculoides* mat is the main factor causing the reduction of  $\text{Fe}^{+++}$  ions to ferrous ( $\text{Fe}^{++}$ ) ions which are highly soluble and thus readily available to *A. filiculoides* plants. Thus, mat formation is an important means by which *A. filiculoides* increases the nutrient status of the underlying water thereby overcoming nutrient limitation and increasing its growth potential.

In the *Azolla-Anabaena* symbiosis, *A. azollae* fixes atmospheric nitrogen while the fern component absorbs and reduces  $\text{NO}_3\text{-N}$  to  $\text{NH}_4\text{-N}$  via the nitrate-nitrite reductase system. Prior to the development of a multi-layered *Azolla* mat under natural conditions, the average concentration of combined nitrogen in the stream water is too low to induce maximum nitrate reductase activity in *Azolla*. Under these conditions, nitrogen assimilation via algal nitrogen fixation occurs far more rapidly than the uptake and reduction of  $\text{NO}_3\text{-N}$  by the fern, with the result that virtually all the nitrogen required by the fern-alga association is provided by the alga. However, during the development of a multi-layered *Azolla* mat, decomposition of the lower mat layers gives rise to higher concentrations of combined nitrogen in the underlying water. This in turn causes a dramatic decrease in nitrogen fixation rates, and at the same time fulfills the substrate induction requirement for nitrate reductase. Thus, after the development of an *Azolla* mat, the increased uptake of combined nitrogen from the underlying water compensates for the loss of fixed atmospheric nitrogen and provides a far larger proportion of the nitrogen required by the symbiotic association. The possession of these two pathways of nitrogen assimilation therefore confers a considerable competitive advantage to symbiotic *Azolla* plants.

Laboratory experiments show that the rates of growth and nitrogen assimilation by *A. filiculoides* are promoted by moderate light intensities (30 to 45 kLux) and water temperatures up to  $27,5^\circ\text{C}$ , but that extremes of light intensity and temperature are inhibitory. The highest  $\overline{\text{RGR}}$  values recorded in the laboratory were always much higher than those

recorded in the field, though light and temperature conditions were comparable. This discrepancy can be attributed to nutrient limitation in the field. Laboratory experiments carried out under both sterile and non-sterile conditions indicate that adequate acclimatization of the *A. filiculoides* material prior to experimental use is of paramount importance.

The reported responses of different species and populations of *Azolla* to environmental factors have been highly variable, usually due to differences in culture conditions (e.g. Johnson *et al.*, 1966; Brotonegoro and Abdulkadir, 1976; Talley *et al.*, 1977; Peters *et al.*, 1980; Talley and Rains, 1980). Therefore, caution is required when the results of this study are compared with those of other workers. The highest growth rates measured during this investigation (32 to 35 % $\cdot$ day<sup>-1</sup>) are comparable to, but lower than, the maximum *Azolla* growth rates measured by Peters *et al.* (1980). These growth rates represent doubling times of 2,0 to 2,2 days and are also comparable to the highest growth rates reported by Blackman (1960) for *Salvinia natans* (37 to 45 % $\cdot$ day<sup>-1</sup>).

Based on the results of controlled environment studies, it may be predicted that rates of growth and nitrogen assimilation in the field would be greatest when midday temperatures ranged between 25 and 35 °C. These conditions are characteristic of the months September to November, and the field studies have confirmed that growth and nitrogen fixation rates are indeed highest during this period of the year. The wide range of temperatures tolerated by *A. filiculoides* permit the plants to survive sub-optimal winter temperatures, particularly when water pH is high, without undergoing the "catastrophic senescence" reported by Holst and Yopp (1979a).

High growth rates in *A. filiculoides* are only attained when nitrogen assimilation rates are high enough to maintain the plant nitrogen content. Under favourable conditions which promote rapid growth rates, nitrogen fixation is far more efficient than nitrate reduction in maintaining the nitrogen content of the fern-alga association. However, despite the protection afforded by the fern, the algal nitrogenase system is more susceptible to unfavourable environmental conditions than the fern's

nitrate-nitrite reductase system. Thus, the nitrogen content of the symbiotic association is possibly maintained by nitrate reduction via the fern when conditions are unfavourable for algal nitrogen fixation and growth rates are low.

The main environmental factors influencing the *Azolla filiculoides*-*Anabaena azollae* symbiosis are summarized diagrammatically in Figure 59. *Azolla filiculoides* is heterosporous and develops both mega- and microsporangia during the sexual phase of its life cycle. The observations made during this study that *A. azollae* maintains its association with *A. filiculoides* via the megasporocarp confirm the earlier report by Huneke (1933). Algal akinetes trapped under the megaspore indusium cap "infect" the new sporophyte apex on germination, thus continuing the fern-alga association throughout the *Azolla* life cycle.

The investigation into the environmental regulation of sporocarp production and germination undertaken during this study provides a new insight into the adaptation of *A. filiculoides* to its environment, since these aspects of the *Azolla* life cycle have not been studied by previous workers. It can be predicted from the results of laboratory experiments that sporocarp production in the field is greatest during the summer months and absent during winter. The field studies confirmed this and also emphasized the importance of mat formation for sporocarp production.

Sporocarps show a high degree of resistance to desiccation at a wide range of temperatures and are thus able to survive seasonal desiccation in the mud of dried-out streams and recolonize the water once the pools have been refilled. In addition, sporocarps are able to withstand a far greater degree of turbulence than mature *A. filiculoides* plants, permitting them to survive the summer floods. They are thus able to serve as a source of infestation for localities further downstream and probably account for the spread of *A. filiculoides* from the study area to a point some 450 km down the Orange River in the Uppington area.

This autecological investigation has shown that the formation of multi-layered mats by *A. filiculoides* is a key feature of its growth potential since it permits the fern to survive unfavourable environmental conditions

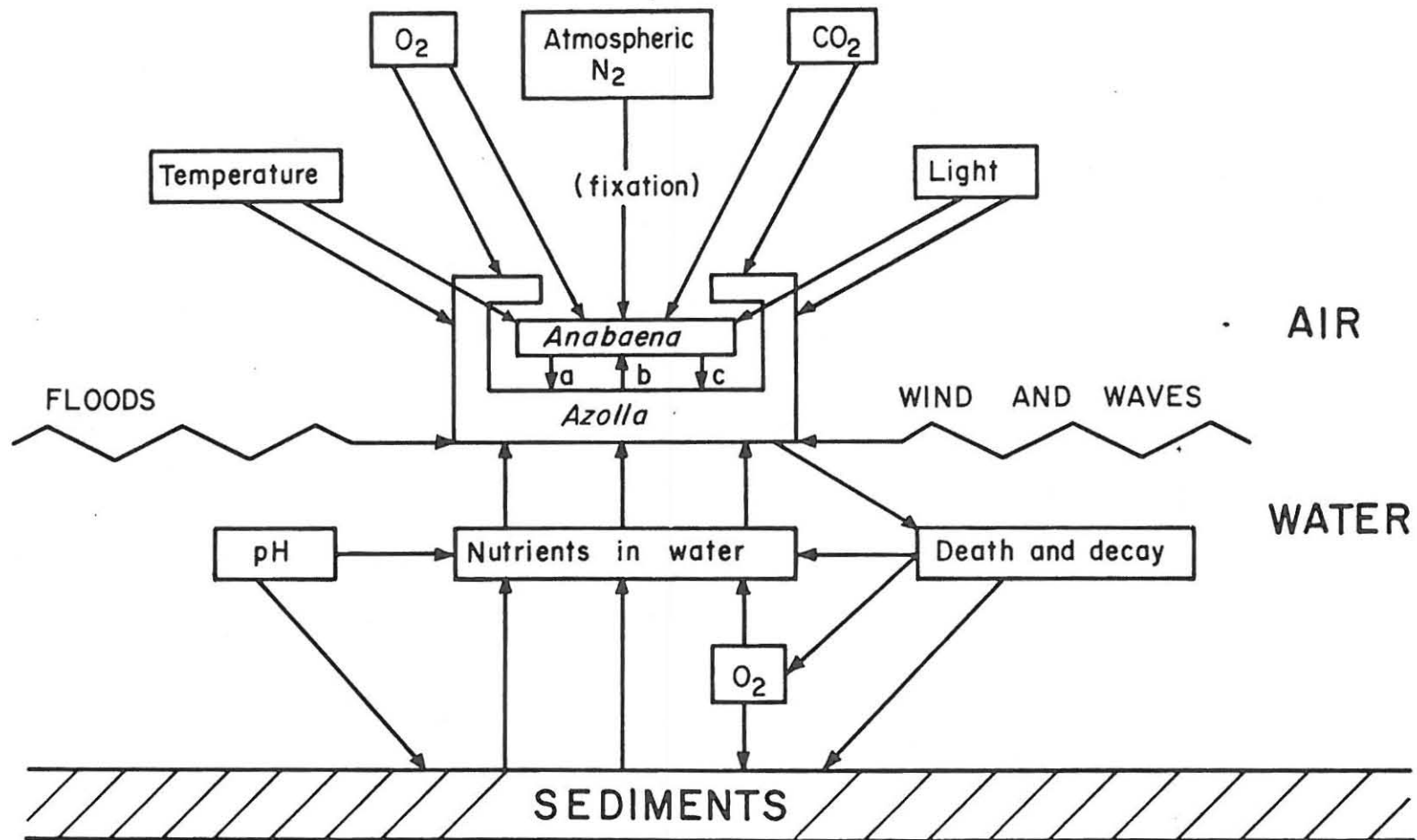


FIG. 59 *Diagram of the main environmental factors influencing the Azolla filiculoides - Anabaena azollae association (after Ashton & Walmsley, 1976). [ a = nitrogenous compounds; b = nutrients; c = growth-promoting substances ].*

in the Hendrik Verwoerd Dam catchment area. Mat formation enables *A. filiculoides* to modify its environment to its own advantage, by affording a degree of thermal insulation, dampening the effects of mild turbulence and increasing the nutrient concentration of underlying waters. Mat formation is also essential for *A. filiculoides* spore production and therefore plays a key role in the continuation of its life cycle as well as its spread to new areas. \*

This study has also shown that the open waters of the Hendrik Verwoerd Dam are not suitable for colonization by *A. filiculoides* and that the fern will therefore never become a problem on the lake. The scarcity of suitable habitats and the regulatory effects of the prevailing hydrological regime accounts for the restricted distribution of *A. filiculoides* in the Hendrik Verwoerd Dam catchment area. Those localities which contain *A. filiculoides* populations are, on the whole, economically unimportant and further control of the plant beyond that exercised by hydrological influences is considered unnecessary. However, in the event of total eradication becoming necessary, other control measures would have to be considered. At present, no herbicide selective for *Azolla* has been found and a chemical eradication programme using a general herbicide would seriously affect other members of the aquatic biota. In this respect, therefore, further research on improved methods of physical, chemical and biological control is necessary.

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APPENDIX I: Calculation of the number of plant samples that need to be collected to ensure sample mean is within 5 % of population mean.....

A		B		C	
90	89	58	55	161	157
104	103	67	64	143	148
93	102	62	63	155	139
98	91	58	65	139	160
91	99	68	59	157	142
90	108	62	56	160	168
97	104	68	60	164	141
89	97	58	62	148	157
108	98	57	64	141	140
104	99	55	60	138	166
109	108	60	62	142	158
101	95	57	58	139	141
94	93	59	60	158	168
107	98	67	57	168	139
89	107	61	59	136	158
89	99	60	64	145	142
110	98	64	61	138	156
92	101	54	67	146	140
100	93	63	55	151	148
110	108	65	61	149	139
$\bar{x}$ = 98,9		60,9		149,9	
x = 6,8		3,8		10,4	
n = 40		40		40	

using: 
$$N = t_{(0,05)}^2 \cdot \frac{S^2}{(D \cdot \bar{x})^2}$$

where:

N = Number of plant samples that should be collected

S = Standard deviation of sample mean

D = Required degree of precision of mean

t = Student's t value for 5 % probability and 39 (n-1) degrees of freedom (= 2,02)

<u>A</u>	<u>B</u>	<u>C</u>
N = 7,72	6,35	7,86

APPENDIX II

\*P.J. ASHTON

Reprinted from:

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ABSTRACT

The growth rate and nitrogenase activity of *Azolla filiculoides* Lam., grown in a wide range of light and temperature regimes, varies with the pH of the nutrient solution used. The degree of cold tolerance varies with plant size and pH of the solution. Annual variations in plant numbers are discussed and evidence is presented indicating that *Azolla filiculoides* is unlikely to colonize the open waters of the Hendrik Verwoerd Dam.

INTRODUCTION

*Azolla* is a genus of small, elegant, aquatic ferns with a world-wide distribution, (Svenson, 1944). The plant consists of a short, branched, floating rhizome with small, alternate, overlapping leaves and has roots which hang down into the water. The genus is of interest in that its members are capable of assimilating atmospheric nitrogen by means of the symbiotic blue-green alga *Anabaena azollae* present in cavities in their leaves. The genus *Azolla* is related to *Salvinia*, and like *Salvinia* is able to rapidly colonize open water surfaces.

Prior to the present study, very little quantitative work had been carried out on the effects of light and temperature on the growth of *Azolla* plants. Ahmad (1941, 1943) found that in laboratory trials, the growth of *Azolla filiculoides* at 25 - 30°C did not occur until the light intensity exceeded 500 Lux. Benedict (1923) indicates that a related species, *Azolla caroliniana* Willd., has considerable resistance to low temperatures.

Nickell (1958, 1961) found that small plants of *Azolla caroliniana* grew very slowly and that the highest growth rates for large plants were achieved at pH 4 - 6.5 at low light intensities.

Some tributary streams in the catchment area of the Hendrik Verwoerd Dam contain large populations of *Azolla filiculoides*, (Figure 1). It was initially feared that like its relative *Salvinia*, *Azolla filiculoides* might colonize the open waters of the newly-formed dam. The present study forms part of an ecological investigation of *Azolla filiculoides* and attempts to account for the limited distribution of the plants.

MATERIALS AND METHODS

Population estimates of *Azolla filiculoides* were carried out monthly at each of twelve sampling sites. A square wooden tube of 100 cm<sup>2</sup> cross-sectional area was used to obtain samples of plants for counting. All estimates of plant density were made on averages of five samples.

In the laboratory, *Azolla filiculoides* was cultured on the ASM-1 medium of Gorham et al., (1964). Large controlled-environment rooms were used to give a wide range of light and temperature conditions. Illumination at low light intensities was by means of cool white fluorescent tubes whilst high light intensities were achieved by using a bank of fan-cooled mercury-vapour photo-flood lamps at varying distances. Where adjustment of the culture solution pH was necessary, this was accomplished by using either 1 N HCl or 1 N NaOH. The solutions were replaced daily by siphoning to avoid disturbance of the plants and to ensure constant nutrient supply. In agitation tests, *Azolla filiculoides* plants were cultured in 300ml. Erlenmeyer flasks containing 100ml. of nutrient solution and shaken on a Bühler rotary shaker with an orbit radius of 3cm.

a) Growth Analysis

Plants of *Azolla filiculoides* were acclimatized in large asbestos trays, under the required light and temperature conditions, for three weeks prior to analysis.

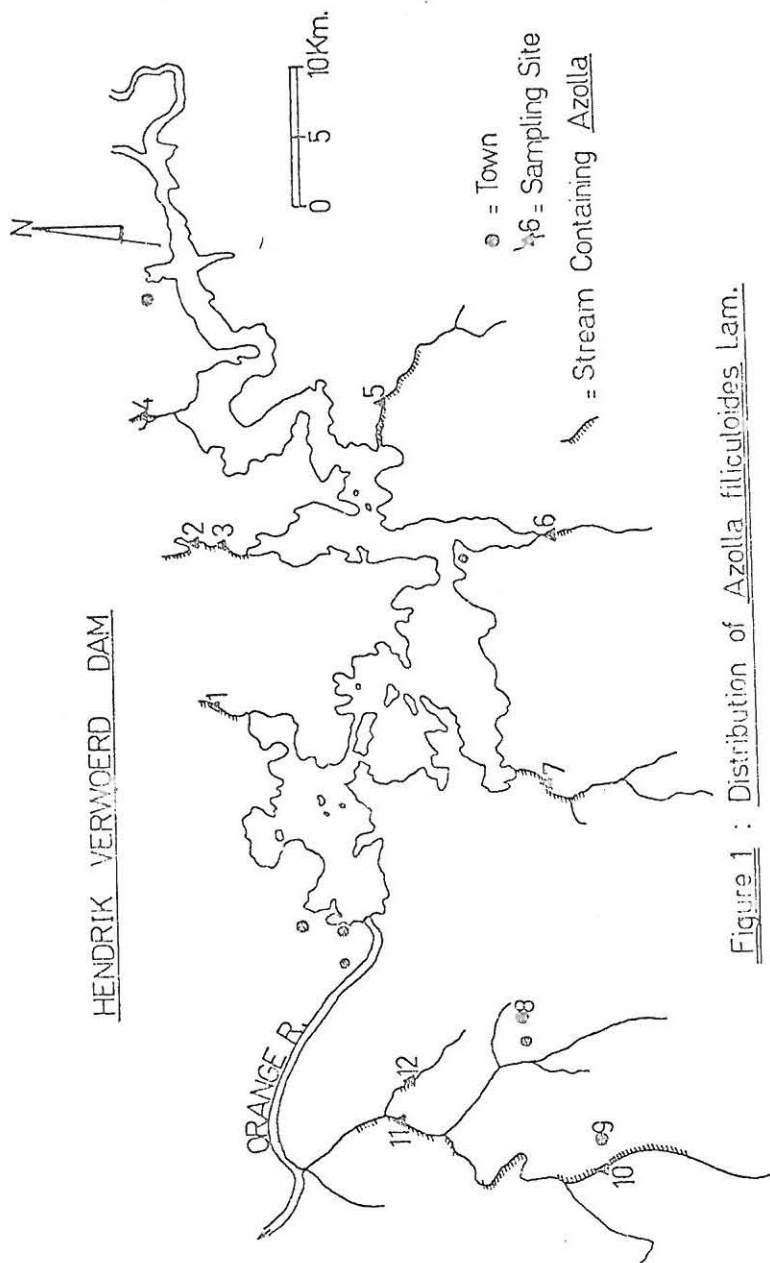


Figure 1 : Distribution of Azolla filiculoides Lam.

For the analysis of growth rate, weighed samples (2,0g.) were placed in 10cm. diameter black-painted plastic dishes containing nutrient solution and reweighed seven days later. The relative growth rate (R.G.R.) was calculated using dry weights and was based on the formula of Blackman (1968):

$$R.G.R. = \frac{(\text{Log}_e \text{ Final wt.} - \text{Initial Log}_e \text{ wt.})}{\text{Time interval (days)}}$$

Results were expressed as grams increase per gram starting weight per day.

b) Nitrogenase Activity

Nitrogenase activity was determined using the acetylene reduction assay of Hardy et al., (1968). Weighed samples (0,2g.) of acclimatized plant material were placed in 25ml. reaction flasks fitted with rubber septa. The flasks were flushed for 4 minutes with a mixture of 0,2atm. acetylene and 0,8atm. of a mixture of argon, oxygen and carbon dioxide in the ratio 77,665 : 22,3 : 0,035 using a Matheson model 665 gas proportioner with needle valve control. The reaction flasks were then placed in constant environment rooms under the required experimental conditions.

Triplicate 50 microlitre samples of the gas phase in each flask were withdrawn after 15 to 30 minutes and analysed. Ethylene formation was detected by gas chromatography, using a Perkin-Elmer 990 gas chromatograph equipped with a flame-ionization detector and fitted with a 6 foot long, 1/8 inch diameter stainless steel column containing 80/100 mesh Porapak N. All analyses were carried out in triplicate at 50°C. High purity nitrogen was used as the carrier gas at a flow rate of 35ml./min. Results were expressed as micromoles of ethylene formed per gram fresh weight per hour.

RESULTS AND DISCUSSION

The growth rate and nitrogenase activity of *Azolla filiculoides* change with increasing light intensity, (Figure 2). Growth increases with increasing light intensity to a maximum in 50% sunlight. Further increases in light intensity decrease growth. In 25% sunlight growth is higher than in full sunlight. Nitrogenase activity is highest in 50% sunlight and fractionally lower in 25% sunlight. In 12,5% sunlight and full sunlight, nitrogenase activity is low. These results indicate that *Azolla filiculoides* grows best in lightly shaded situations and nitrogenase activity may depend on photosynthetic rates at low light intensities.

Both the relative growth rate and nitrogenase activity of *Azolla filiculoides* increase with increasing daylength, (Figure 3), up to 8 hours. With daylengths longer than 8 hours, the nitrogenase activity does not increase while the growth rate increases. The results show that though the total amount of nitrogen fixed during an 8 hour day is less

Figure 2: The relative growth rate (R.G.R.) and nitrogenase activity of *Azolla filiculoides* plants at different light intensities.

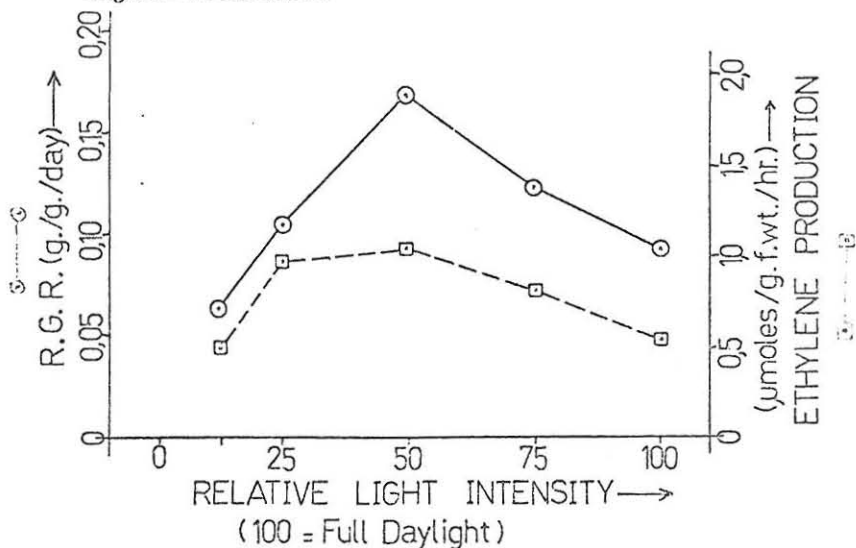
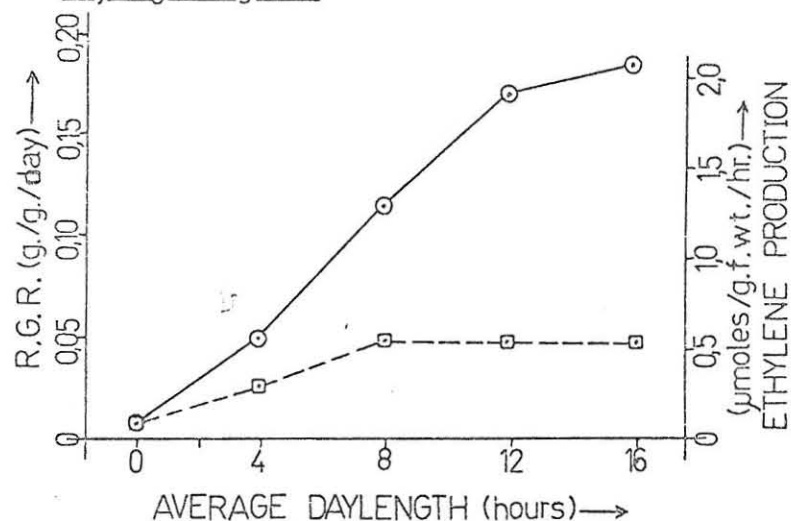


Figure 3: The relative growth rate (R.G.R.) and nitrogenase activity of *Azolla filiculoides* plants under different daylength regimes.



than that fixed during a 16 hour day, it is still sufficient to support the growth of *Azolla filiculoides* plants during periods of short daylength at a level approximately equivalent to two-thirds of the growth rate of plants grown under 16 hours daylight.

With increasing light intensity, the relative growth rate (Figure 4) and nitrogenase activity (Figure 5) increase at all of the temperatures used, though the increase is minimal at the extremes of temperature. The 'optimum' temperature for growth and nitrogenase activity also increases with increasing light intensity (a,b,c). However, the higher the light intensity, the smaller is the degree of change in 'optimum' temperature, indicating that the 'optimum' temperature changes only at low light intensities and stabilizes at high light intensities. Ahmad (1941) found that maximum growth of *Azolla filiculoides* occurred at 20 - 21°C at the very low light intensities of 500 to 2000 Lux. This suggests that at very low light intensities, the 'optimum' temperature does not change until the light intensity is in the region 2000 to 5000 Lux.

Figure 4: The effect of temperature on the relative growth rate (R.G.R.) of *Azolla filiculoides* at different light intensities.

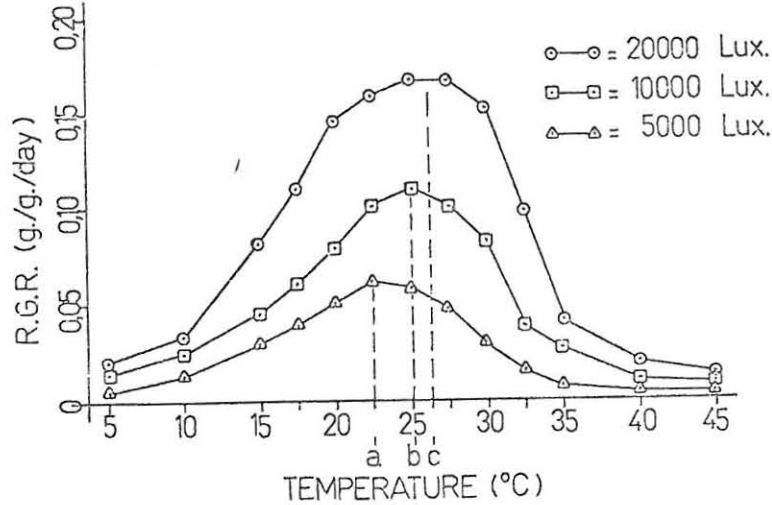
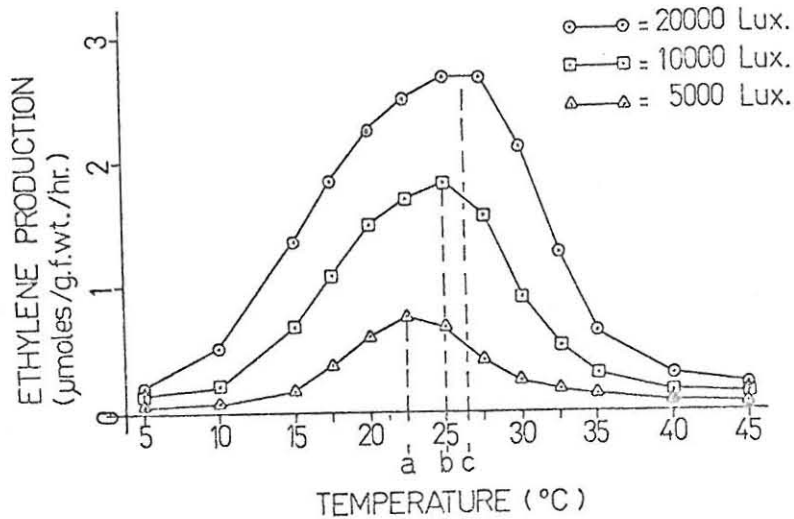


Figure 5: The effect of temperature on the nitrogenase activity of *Azolla filiculoides* at different light intensities.



Temperatures below 10°C and above 35°C decrease growth and nitrogenase activity to a minimum. The increase in nitrogenase activity with increasing light intensity, again suggests that at the low light intensities used, the rate of fixation of nitrogen may be dependent on the rate of photosynthesis.

Plants of *Azolla filiculoides* occurring in the tributary streams of the Hendrik Verwoerd Dam experience very low temperatures during the winter months. The degree of cold tolerance shown by different sized plants *Azolla filiculoides* was determined by varying their length of exposure to 5°C, (Figure 6). Short periods of low temperature do not reduce the number of viable plants. Longer exposures markedly decrease the number of viable small plants (5mm. diameter), but cause only a slight decrease in the number of viable large plants.

The percentage germination of megaspores is markedly decreased by even short exposures to low temperatures, (Figure 6). Under natural conditions, megaspores are formed in the summer months (September to January) and most of these germinate before March. The degree of cold tolerance shown by megaspores will therefore only apply to those which germinate in the late autumn and early winter months.

The degree of cold tolerance shown by large (20mm diameter) plants of *Azolla filiculoides* varies with the pH of the solution (Figure 7). At the alkaline pHs of 8 and 10, the number of viable plants is only slightly decreased after 30 days of exposure to 5°C. The number of viable plants drops with decreasing pH and lengthening exposure times. The pH of the streams containing *Azolla filiculoides* in the catchment area of the Hendrik Verwoerd Dam varies from 7,5 to 9,8. Therefore, even at the lowest pH experienced by *Azolla filiculoides* in the field, the plants are well able to withstand long exposures to low temperatures, agreeing with the observations of Benedict (1923) on *Azolla caroliniana*.

Figure 6: The % megaspore germination and survival (% viable plants) of different sizes of *Azolla filiculoides* plants after varying lengths of exposure to 5°C.

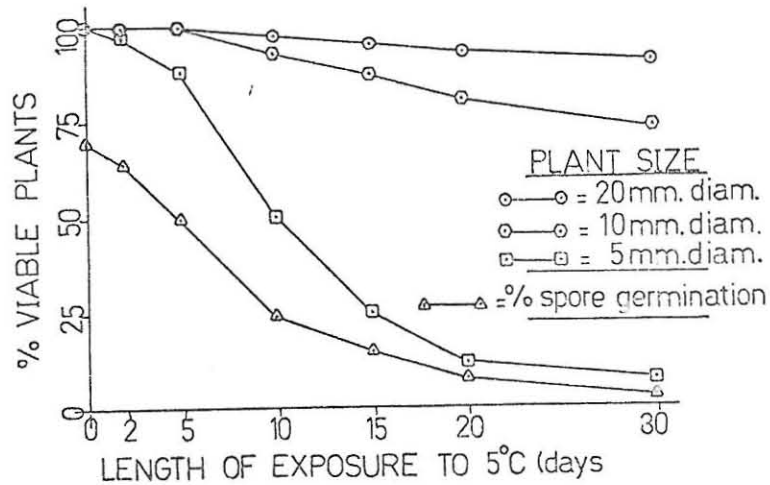
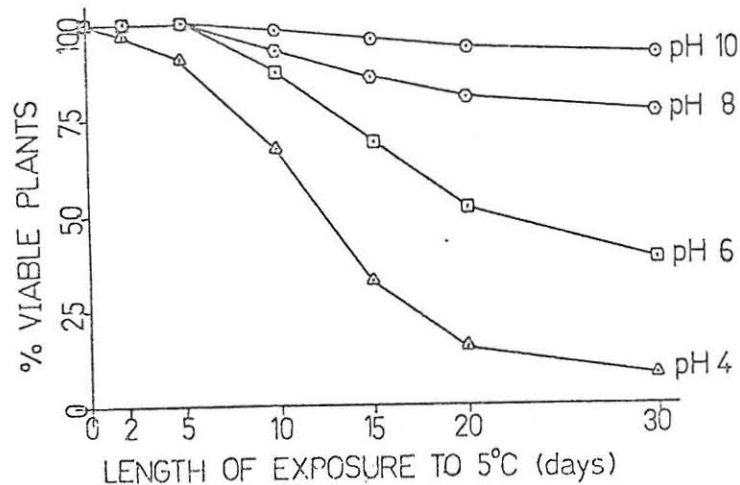


Figure 7: The survival (% viable plants) of *Azolla filiculoides* plants in different pHs, after varying lengths of exposure to 5°C.



In *Azolla filiculoides* plants, the uptake of combined nitrogen is greatest in the acidic range and very low in the basic range, while the fixation of atmospheric nitrogen is highest in the basic range and almost non-existent in the acidic range. The interaction of these two systems therefore ensures the nitrogen supply of the plants over a wide range of pHs. Figure 8 demonstrates the effects of different light intensities on the growth rate of *Azolla filiculoides* grown in solutions of differing pH, when supplied with a source of combined nitrogen in the form of nitrate at 10 ppm.

At a low light intensity of 15000 Lux, growth is greatest in the acidic range of pH 5 - 6, with a second, lower growth peak in the basic range of pH 9 - 10. This agrees with the results of Nickell (1961) who showed maximum growth of *Azolla caroliniana* at pH 4, 5, 6 when grown under very low light intensities. With increasing light intensities up to 60000 Lux, growth in the acidic range decreases while growth in the basic range increases to a maximum. At a higher light intensity of 75000 Lux, growth decreases in both the acidic and basic ranges. The results show that the range of pHs found in the tributary streams of the Hendrik Verwoerd Dam are suitable for maximum growth of *Azolla filiculoides* and that very high light intensities inhibit growth.

In undisturbed optimal conditions, large plants of *Azolla filiculoides* grow exponentially until the available surface area is covered. Once the surface of the water is completely covered, the rate of growth and nitrogenase activity decrease markedly with time as the plant density increases, (Table 1).

TABLE 1: The effect of increasing plant density on the relative growth rate (R.G.R.) and nitrogenase activity of *Azolla filiculoides* with time

Time elapsed (days)	R.G.R. (g./g./day)	Ethylene Production (μmole/g.f.wt./hr.)	Av. Plant density (no.plants/dm <sup>2</sup> )
0	0,2394	3,14	3,18
5	0,2378	2,89	10,53
10	0,2349	2,61	34,58
15	0,0534	1,16	52,46
20	0,0319	0,27	68,74

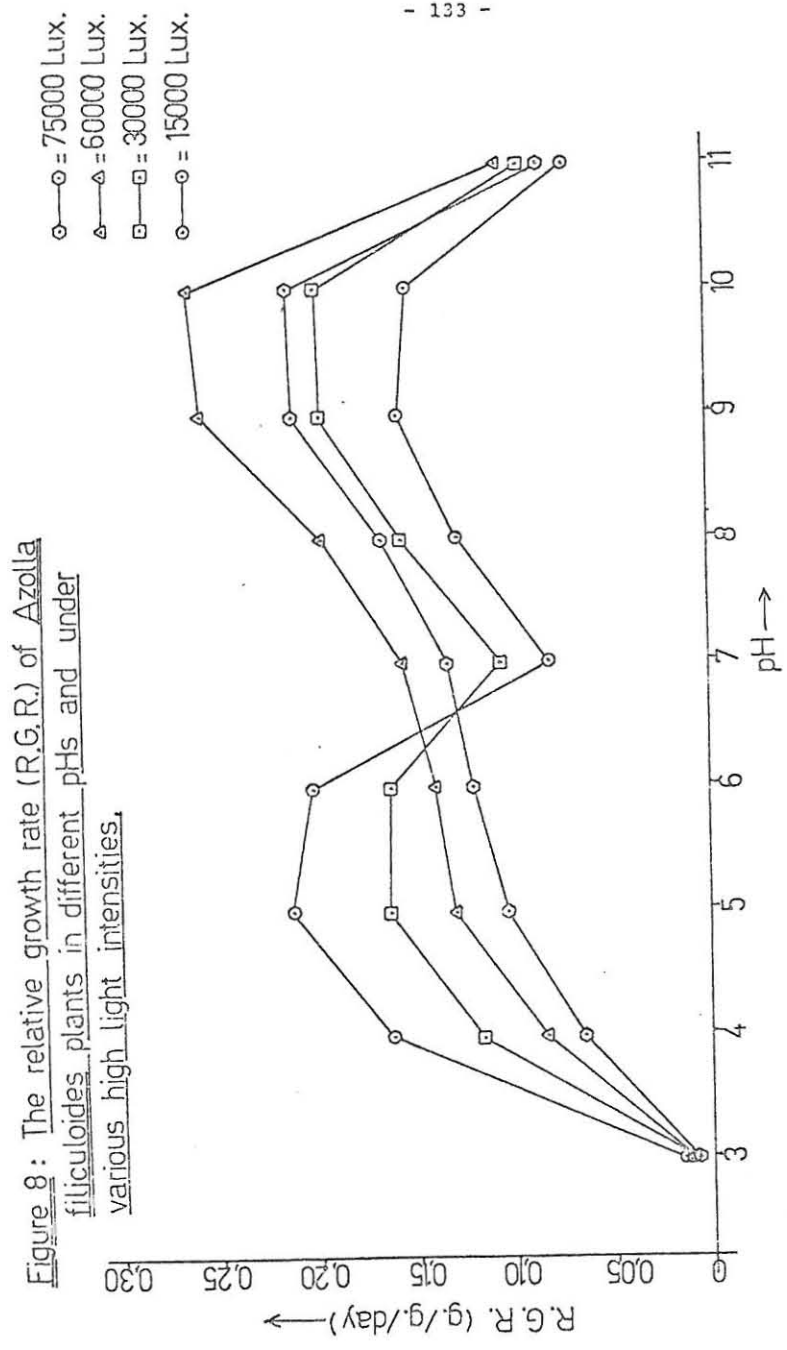


Figure 8: The relative growth rate (R.G.R.) of *Azolla filiculoides* plants in different pHs and under various high light intensities.

Figure 9 shows a histogram of average plant density at a typical riverine sampling site. The peaks show the large numbers of plants present during summer, (September to January). The growth rate of these plants is very low since the water surface is completely covered with plants. The drop in plant density during the months January to April is due to flood action removing almost all of the plants. May to July is the period of greatest growth. Here, the remaining *Azolla filiculoides* plants, having a high degree of cold tolerance at alkaline pHs, rapidly colonize undisturbed water surfaces after the flooded streams have returned to normal.

Nickell (1958) described low growth rates in small plants of *Azolla caroliniana*. Similarly, small plants of *Azolla filiculoides* (2mm. diameter) have very much lower growth rates and nitrogenase activity than larger plants, (Table 2).

TABLE 2: The relative growth rate (R.G.R.) and nitrogenase activity of different sized plants of *Azolla filiculoides*

	Plant size (diameter)			
	2mm.	5mm.	10mm.	20mm.
R.G.R. (g./g./day)	0,0220	0,0420	0,1131	0,1443
Ethylene Production (u moles/g.f.wt./hr)	0,18	0,30	1,29	1,71

Plants of *Azolla filiculoides* fragment very easily, the degree of fragmentation depending on the severity of agitation or shaking. High shaking rates, corresponding to small waves on an open lake surface, result in very small plant fragments with low growth rates and nitrogenase activity, (Table 3).

Figure 9: Histogram of average plant density at a typical riverine sampling site, (station 11), from February 1971 to May 1974.

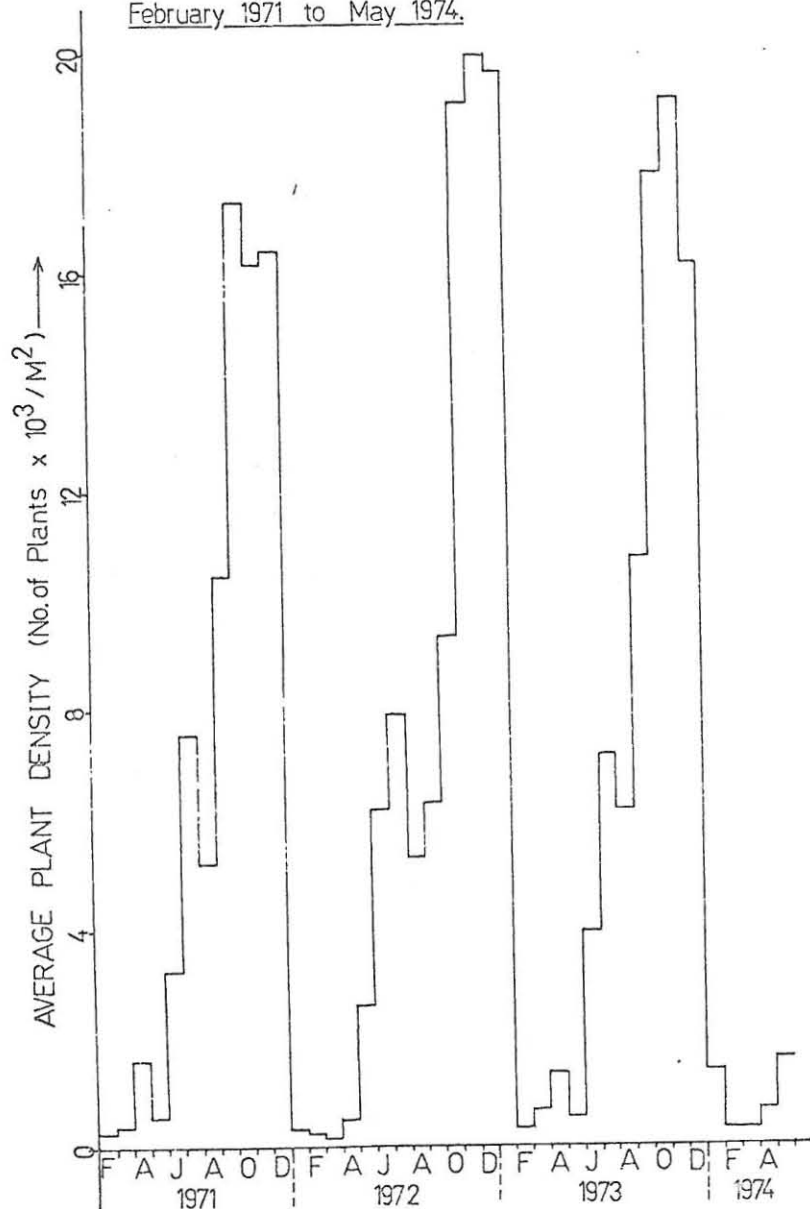


TABLE 3: The effect of agitation on the relative growth rate (R.G.R.), nitrogenase activity and resulting fragment size of *Azolla filliculoides* plants

Shaking rate (revs./min.)	R.G.R. (g./g./day)	Ethylene production ( $\mu$ moles/g.f. wt./hr.)	Av. Fragment size (mm. diameter)
5	0,1422	1,69	18
25	0,1236	1,44	15
75	0,0410	0,31	5
125	0,0230	0,19	2

During seasonal flooding of the tributary streams of the Hendrik Verwoerd Dam, most of the aggregations of *Azolla filliculoides* plants are broken up and washed downstream into the open waters of the lake where the plants are very soon further fragmented by wave action. These plant fragments are carried by surface currents onto the leeward shores of the lake where yet further fragmentation results from the continued abrasive action of waves on the shoreline.

The survival rate of very small fragments of *Azolla filliculoides* plants is considerably reduced with increasing lengths of exposure to high light intensity, (Table 4).

TABLE 4: The survival (% viable plants), of 2mm. diameter plants of *Azolla filliculoides* after varying lengths of exposure to high light intensity, under different daylength regimes. (pH 9, 75000 Lux)

Length of Exposure (days)	8 hour day (% viable plants)	16 hour day (% viable plants)
5	68	54
10	43	27
15	20	9
20	4	0
25	0	0

At high light intensity, the decrease in number of viable *Azolla filiculoides* plants grown under 16 hours daylight is greater than that of plants grown under 8 hours daylight. In both daylength regimes, 2mm. diameter plants of *Azolla filiculoides* soon succumb to high light intensity. Throughout the year, light intensities at the Hendrik Verwoerd Dam are very high, of the order of 80000 to 115000 Lux. Therefore, plants of *Azolla filiculoides* washed into the lake by floods and fragmented by wave action will be unable to survive for longer than three weeks, regardless of when the flooding occurs.

It is evident from the results presented, that although *Azolla filiculoides* will not become a problem on the exposed waters of the Hendrik Verwoerd Dam, it will continue to flourish in the tributary streams until an efficient method of eradication is found.

#### ACKNOWLEDGEMENTS

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# The aquatic fern *Azolla* and its *Anabaena* symbiont

P. J. Ashton and R. D. Walmsley

The aquatic fern *Azolla* contains a symbiotic, heterocystous, blue-green alga, *Anabaena azollae*, within cavities of its leaves. By the process of nitrogen-fixation the alga is capable of fulfilling the nitrogen requirements of the association. Because the fern can form dense mats on water surfaces, it is classified as a water weed in many areas. Research has indicated that the growth of the fern-alga association is influenced by numerous interacting environmental and physiological factors.

## Introduction

*Azolla* is a genus of small aquatic ferns with a wide distribution [1]. Strasburger, in 1873, first observed that the dorsal lobes of the leaves on *Azolla filiculoides* contained cavities in which a heterocystous blue-green alga, *Anabaena azollae*, could always be found [2]. This fern-alga association was subsequently shown to be capable of sustaining growth on nitrogen-free media [3]. These unique characteristics stimulated many studies on the fern-alga association in the early part of the twentieth century. Most of these investigations proved inconclusive in elucidating the interdependence of the two organisms [2].

*Azolla* is of interest not only as a botanical curiosity, but through its importance in nature, both as a weed and as a fertilizer. In areas where water resources are limited, the growth of aquatic plants has caused some concern. Of particular interest are free-floating plants such as *Azolla*, *Salvinia*, and *Eichhornia* which are able to colonize areas of open water. These plants can form dense mats over water surfaces on which terrestrial plants are able to grow to form a type of sudd vegetation [4, 5]. This is undesirable since the decreasing area of open water imposes limitations on water usage. Unlike *Salvinia* and *Eichhornia*, *Azolla* does not require the presence of a source of combined nitrogen in the waters where it occurs, since the fern can fulfil its nitrogen requirements through the assimilation of atmospheric nitrogen by its algal symbiont. Potentially, therefore, *Azolla* can easily colonize water bodies which are deficient in nitrogen and unsuitable for growth of other aquatic plants.

Knowledge of the physiological and ecological factors influencing the growth of the fern is therefore essential in order to predict the setting in which it may become a problem. *Azolla* has now become the subject of increased study on a global basis [6-12].

## The fern-alga association

An *Azolla* plant consists of a short, branched, floating stem, bearing roots which hang down into the water. The stem and branches are covered with small, alternate, overlapping leaves (figure 1). Each leaf is bilobed, the upper lobe contains the green pigment chlorophyll, whilst the lower lobe lacks chlorophyll and is colourless. Under certain conditions, an anthocyanin pigment also occurs in the upper lobes of the leaves, giving the fern a reddish-brown colour. The alga, *Anabaena azollae*, is closely associated with the apical meristem of the fern

(figure 2 (A)) and grows in unison with the fern. As leaf primordia are formed by the meristem, filaments of the alga become entrapped within the developing cavities in the dorsal lobes of each leaf (figure 2 (B)). The alga is restricted to this micro-environment during its growth and dies off when the leaf senesces. It has been estimated that in *Azolla caroliniana* the alga contains from 7.5 to 15 per cent of the total chlorophyll of the association [6].

The fern is heterosporous and develops both mega- and microsporangia during the sexual phase of its life cycle. The alga maintains its association with the fern during sexual reproduction. Several workers have reported that the alga maintains its link with the fern by being associated with both the microsporangia and megasporangia [13, 14, 15], whilst others report that a link with the megasporangium only is maintained [16]. Studies on *A. filiculoides* by the authors have shown that algal akinetes are found only in the megasporangium, embedded in a cavity under the indusium cap (figure 3). After fertilization of the megaspore, a zygote is formed which develops into a sporophyte with its associated alga. The continuity of the association is demonstrated in the life-cycle diagram (figure 4).

In order to assess the interdependence shown by the component organisms of a symbiotic association, one must isolate the individual organisms and then recombine them after establishing their individual growth requirements. The literature to date shows that the fern and algal components have been isolated [16, 17], but there are no reports of successful recombination. *Anabaena azollae* has been cultured from *A. pinnata* [18], from *A. caroliniana* [19] and from *A. filiculoides* by the authors. For the authors' cultures, *Anabaena azollae* colonies were isolated by micro-manipulation from the cavities of surface-sterilized *A. filiculoides* leaves and subjected to heat treatment to remove contaminating bacteria [19]. Isolates were then placed on nitrogen-free agar plates for further isolation and growth.

In these cultures of *Anabaena azollae*, the heterocyst

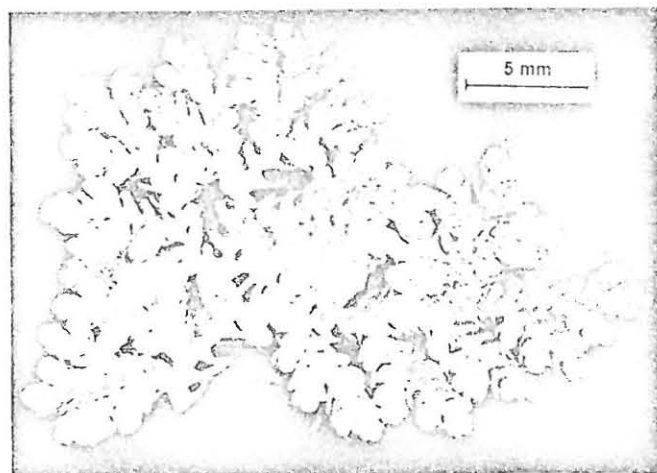


Figure 1 Sporophyte of *A. filiculoides* showing overlapping dorsal leaf lobes and general growth habit.

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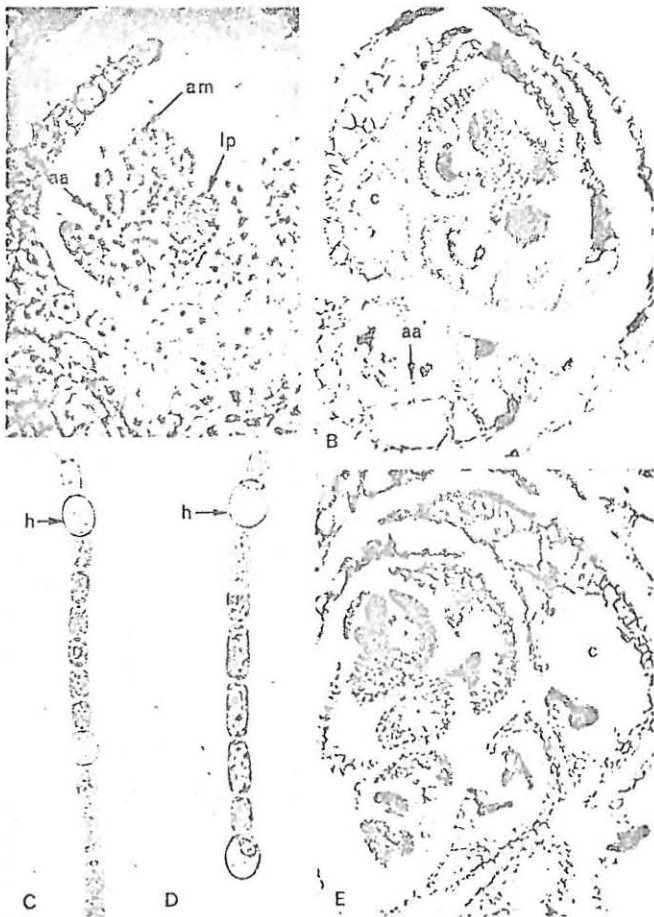
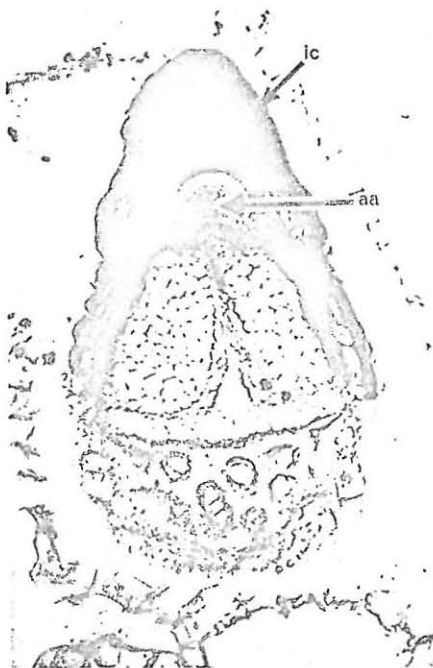


Figure 2 (A)( $\times 100$ ) Apical meristem (am) and developing leaf primordia (lp) of *A. filiculoides* showing the close association with *Anabaena azollae* (aa). (B)( $\times 45$ ) L.S. of *A. filiculoides* showing cavities (c) of dorsal leaf lobes containing colonies of *Anabaena azollae* (aa). (C)( $\times 600$ ) *Anabaena azollae* filament in fern, showing heterocyst (h). (D)( $\times 500$ ) *Anabaena azollae* in culture showing heterocyst (h); [note cells are more elongated than cells in (C)]. (E)( $\times 60$ ) L.S. of alga-free *A. filiculoides* showing empty leaf cavities (c).

Figure 3 (below) ( $\times 70$ ) Megaspore of *A. filiculoides* showing space under the indusium cap (ic) where akinetes of *Anabaena azollae* (aa) are found.



frequency is from 7.0 to 15.5 per cent higher than reported values for free-living species [20] and the vegetative cells are more elongated than those in the leaf cavities of the fern (figures 2 (C) and (D)). These differences in morphology are probably due to the effects of the mineral medium and the artificial culture environment. The lack of previous knowledge on the contribution of the alga to the association is because a large number of blue-green algae are notoriously difficult to culture. No free-living growth forms of *Anabaena azollae* have yet been discovered, although this may be attributed to insufficient taxonomic knowledge of the blue-green algae. Consequently, this has led to doubt and scepticism as to whether any cultured isolate is really the true symbiont, since, apart from the morphological differences, attempts to recombine the cultured *Anabaena* with alga-free plants have failed.

Alga-free plants of *Azolla* have rarely been found in nature [2], but varying degrees of success in freeing *Azolla* of *Anabaena* have been claimed for numerous methods [6, 16, 17, 21]. A convenient and rapid method used by the authors involves the use of antibiotics. *A. filiculoides* may be freed of the alga by subjecting surface-sterilized fronds to three consecutive antibiotic treatments, each of one week's duration. The basal nitrogen-enriched medium is supplemented with (a) 25 mg/l Na penicillin G, (b) 4 mg/l terramycin, and (c) 5 mg/l streptomycin sulphate. The resulting alga-free fern (figure 2 (E)) is more compact in size and shape, grows more slowly than the fern-alga association and also requires a source of combined nitrogen. This demonstrates the importance of the alga for normal growth of the fern and suggests the existence of a growth-promoting substance or substances which are transferred from the alga via the apical meristem and/or the leaf cavities.

Despite the ability of the fern and the alga to exist separately in culture, it is obvious that for normal growth, the fern relies heavily on the presence of the alga. This dependence is largely related to the nitrogen-fixing metabolism of the alga and the transfer of excreted nitrogenous compounds. In turn, the alga, by virtue of its position in the leaf cavities, derives physical protection and all its nutrient requirements (except nitrogen) from the fern. Controversy about the fern-alga association and the interrelationships of the two organisms will continue until the culture and successful recombination of both organisms has been achieved by a single group of workers in the same laboratory.

#### Nitrogen fixation

Although it is well known that heterocystous blue-green algae are capable of nitrogen fixation, only recently was it conclusively proved that *Anabaena azollae* is the agent of fixation in the *Azolla-Anabaena* association [6, 7, 8, 11]. The acetylene reduction assay, a sensitive gas chromatographic technique used for the detection of the nitrogen-fixing enzyme system [22], has proved valuable in elucidating the nitrogen-fixing characteristics of the association. The assay is based on the ability of the enzyme complex which fixes nitrogen to reduce acetylene to ethylene, even in the presence of nitrogen. The rate of acetylene reduction is directly proportional to the rate of nitrogen fixation and is controlled by the same physiological and environmental factors. Evidence gained from using the assay has shown that

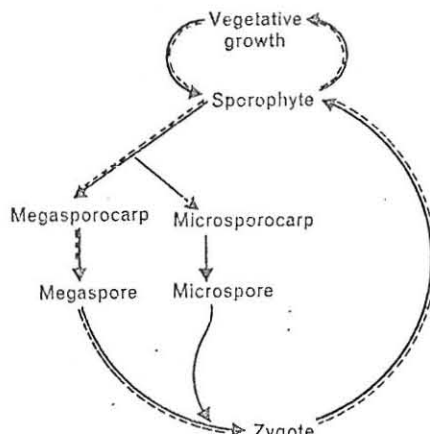


Figure 4 Life cycle of *A. filiculoides* (-) showing the continuation of the association with *Anabaena azollae* (- - -).

the nitrogen-fixing characteristics demonstrated by the association are typical of a blue-green alga [6, 7, 8, 11, 20]. Both the association and the isolated alga are capable of reducing acetylene, whereas the alga-free fern cannot, nor can it sustain growth on a nitrogen-free medium.

Studies by the authors have shown for *A. filiculoides* that there is a close link between growth and acetylene reduction (nitrogen-fixation). This means that if an *Azolla* plant registers a low acetylene reduction rate, then it can be expected that its growth rate will also be low. Under ideal conditions, *A. filiculoides* registers maximum growth and acetylene reduction rates when grown on a nitrogen-free medium. The nitrogen requirements of the association can therefore be more efficiently fulfilled by the fixation of atmospheric nitrogen. This also indicates that considerable amounts of assimilated nitrogen are transferred from alga to fern. The form and manner in which this nitrogen is transferred have not yet been determined. *Anabaena azollae* isolated from *A. filiculoides* excretes large quantities of ammonia into its culture medium. This would point to ammonia being the mobile nitrogen form; however, no information is yet available on ammonia concentrations within the leaf cavities of the fern.

In free-living nitrogen-fixing *Anabaena* species, the ability to reduce acetylene is completely suppressed after several days growth in a medium containing a source of combined nitrogen [24, 25]. This occurs because the enzyme system which reduces acetylene is inhibited by the combined nitrogen in the medium. *Anabaena azollae* in free culture can withstand up to 100 mg/l of ammonium-nitrogen before any appreciable inhibition of acetylene reduction occurs. The metabolism of nitrogen-fixation in *Anabaena azollae* therefore differs from that of free-living *Anabaena* species. In the fern-alga association, a similar situation arises. Although acetylene reduction capacity is considerably suppressed, *A. caroliniana* plants are still capable of reducing acetylene after six to seven months' growth on a medium containing nitrogen [6]. The fern acts as a 'sink' for the nitrogenous compounds excreted by the alga and it is therefore probable that the combined nitrogen in the medium interferes with the fern's action in this respect, since the fern can obtain nitrogen from an alternate source. The accumulation of excreted nitrogenous substances in the alga's micro-environment and a reduction in the growth rate of the fern are two factors which probably lead to this suppression of acetylene reduction, although it is also possible that nitrogenous compounds are transferred from the fern to the alga in this situation.

The nitrogen-fixing characteristics of *Azolla* are therefore unique and demonstrate that either of the two organisms in the association can govern the growth and metabolic activity of the other. This depends largely on the nitrogen status of the medium on which the association is grown.

#### Growth of the fern

An estimate of the potential for *Azolla* to colonize an area of open water requires knowledge of the growth rate of the plant under controlled environmental conditions. Experiments utilizing constant environment chambers have revealed a number of interesting features concerning the growth requirements and nutrition of *Azolla filiculoides*. Because the fern floats on water, it can be influenced by physical and chemical factors from both the air and water phases. In general, however, the fern behaves in much the same way as most green plants, in that it has optimal temperature, light, pH, and nutrient requirements.

*A. filiculoides* is capable of growing within a wide temperature range (5°-45°C), but has an optimum

temperature of approximately 27.5°C [10]. Light plays a major role in governing the growth of the fern largely through its effects on the process of photosynthesis. The light-dependent reactions of photosynthesis produce 'carbon skeletons' and high-energy substrates which are essential for the metabolic activities of the fern and its symbiont. The action of light is further complicated by the fact that light intensity and pH of the medium have interacting effects on the growth rate of the fern [10]. *A. filiculoides* grows maximally at low pH (5) when subjected to low light intensity (15 000 lx) and also at high pH (9-10) when grown under high light intensity (60 000 lx). The overall effect of pH and light intensity is demonstrated in figure 5. When the plant experiences light intensities above 60 000 lx, there is an inhibition of growth at all pH values. Since full sunlight is of the order of 80 000 to 115 000 lx, these characteristics indicate that the fern favours environments where a certain degree of shading is available, and it is also able to tolerate a wide range of pH and temperature.

Like most plants, *A. filiculoides* is sensitive to changes and deficiencies in the supply of plant nutrients. For optimal growth, the fern requires all the macro- and micro-nutrients which are essential for normal plant growth [2]. Macro-nutrients such as phosphorus, nitrogen, potassium, calcium, and magnesium are especially important and produce marked effects on growth of the fern if present in too high or too low concentrations. Since nitrogen-fixation by the symbiont also plays a dominant role in regulating the growth of the fern, micro-nutrients such as iron, cobalt, and molybdenum, which have been shown to be essential for the nitrogen-fixing process [2, 21, 23], must also be present in adequate supply in waters where the fern grows.

In the natural habitat of the fern, nutrient availability is governed by the chemical conditions beneath the water surface, particularly the pH and oxygen status. Certain elements may be present in large quantities, but because of unsuitable pH and oxygen regimes, are not available for uptake by the fern. This situation has been exemplified by studies on *A. caroliniana* in small Danish lakes [12]. The fern thrived where waters were anaerobic and iron was present in the ferrous (reduced) form, whereas in lakes where waters were not anaerobic and iron was present in the ferric (oxidized) form, plants became chlorotic and perished on account of iron deficiency.

Growth of *Azolla* is not complete unless a constant circulation of atmospheric gases is maintained over the plant. Oxygen, nitrogen, and carbon dioxide exert their effects through the metabolic processes of photosynthesis, nitrogen fixation, and respiration. Observations on *A. filiculoides* have indicated that this fern grows better in the laboratory when kept in a container which has no

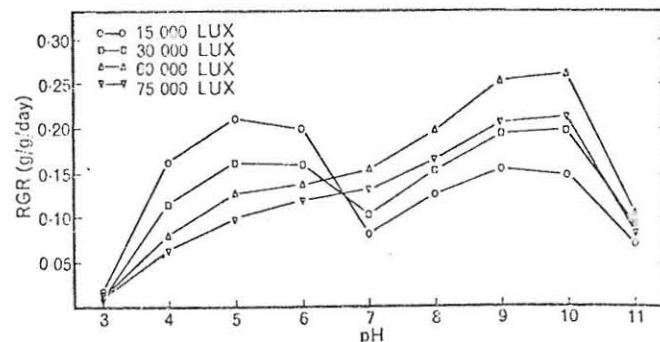


Figure 5 The effect of pH on the relative growth rate (RGR) of *A. filiculoides* grown under different light intensities. (Reproduced by permission of Prof. E. M. van Zinderen Bakker, Sr. [10])

obstructions such as bungs, cotton wool, or aluminium foil placed over the opening of the container. Presumably these structures restrict the diffusion and free passage of atmospheric gases into and out of the container.

*Azolla* plants are very fragile and susceptible to fragmentation if physically disturbed [10]. Fragmentation of *A. filiculoides* plants produces a marked reduction in the growth and acetylene reduction rates of resultant fronds on both nitrogen-free and nitrogen-containing media (Table 1). Therefore on exposed areas of open water where wind and wave action are high and a large degree of turbulence exists, *A. filiculoides* would show poor growth. On such bodies of water the development of *Azolla* mats would be highly unlikely.

**Azolla-environment interaction**

When an *Azolla* mat develops over a water surface and persists for any length of time, it can be expected that drastic changes will occur in the waters underneath the mat. Penetration of light is prevented, sedimentation of organic matter is increased, and there is a physical impediment to the transfer of oxygen from the air into the water. Decomposition processes are therefore encouraged and completely anaerobic conditions may occur.

As previously mentioned, the environment can influence *Azolla* in several ways, for example, through light, turbulence, pH, and oxygen and nutrient availability. An example of an *Azolla* reaction on the environment and in turn the effect of the environment on *Azolla* is illustrated by the results of studies on the Hendrik Verwoerd Dam, South Africa's largest man-made lake. *A. filiculoides*, native to South America, was introduced to South Africa some 30 years ago. It has subsequently formed dense mats on several water systems in South Africa. Several tributary streams of the Hendrik Verwoerd Dam develop mats of *A. filiculoides* and the authorities thought that the plant might become a problem in the dam itself. However, surveys carried out over a period of 40 months have indicated that the fern is of little significance in the dam. Plants which enter the lake via the tributary streams are dispersed and fragmented by wind and wave action and usually die off [10].

In the tributary streams, however, stretches of up to 1.4 km in length can be completely covered with multi-layered mats of *Azolla* for up to six months of the year



Figure 6 Tributary stream of the Hendrik Verwoerd Dam covered with a multi-layered mat of *A. filiculoides*.

(figure 6). Most of these tributary streams are slow-flowing for the greater part of the year and some even dry up into a series of pools. Routine monitoring of the conditions under these mats has shown that the plants in the lower layers of the mats are in a state of decomposition and waters underneath are anaerobic. High levels of nutrients, particularly phosphorus, manganese, iron, and nitrogen are present in the waters due to their liberation from sediments and decaying plants by chemical and bacterial action. The release of these nutrients into the waters provides further stimulus for the growth of *Azolla* plants and the consequent proliferation of the mat. The interacting factors affecting growth of *A. filiculoides* under these conditions are shown diagrammatically in figure 7. The anaerobic, nutrient-rich environment below the mats represents an ecosystem which is restricted to only the hardiest of biota. Fish are unable to survive in the waters and the normal aquatic insect populations are replaced by those which are adapted to a terrestrial or semi-aquatic environment.

Owing to the neo-tropical conditions in the area, growth of these mats can occur throughout the year.

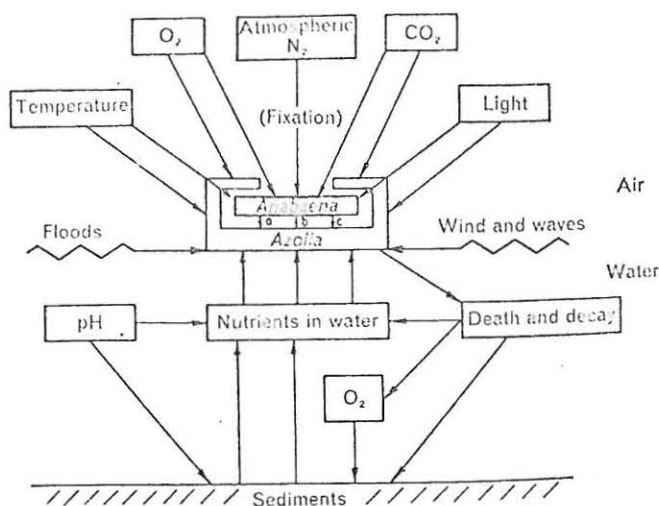


Figure 7 Diagram of the main environmental factors influencing the *Azolla-Anabaena* association. a= nitrogenous substances; b= nutrients; c= growth-promoting substances.

Table 1

The effect of agitation intensity (shaking rate) on the resulting fragment size, relative growth rate (R.G.R.) and nitrogenase activity (measured as the rate of ethylene production per gram fresh weight per hour) of *A. filiculoides* plants grown in nitrogen-free and nitrogen-containing solutions.

Shaking rate (revs/min)	Av. fragment size (mm diameter)	R.G.R.		Ethylene production rate ( $\mu\text{mole/g f. wt./h}$ )	
		+N	-N	+N	-N
1	20	0.1479	0.1603	1.53	1.71
5	18	0.1254	0.1422	1.45	1.69
25	15	0.1102	0.1236	1.27	1.44
75	5	0.0363	0.0410	0.28	0.31
125	2	0.0118	0.0230	0.14	0.19

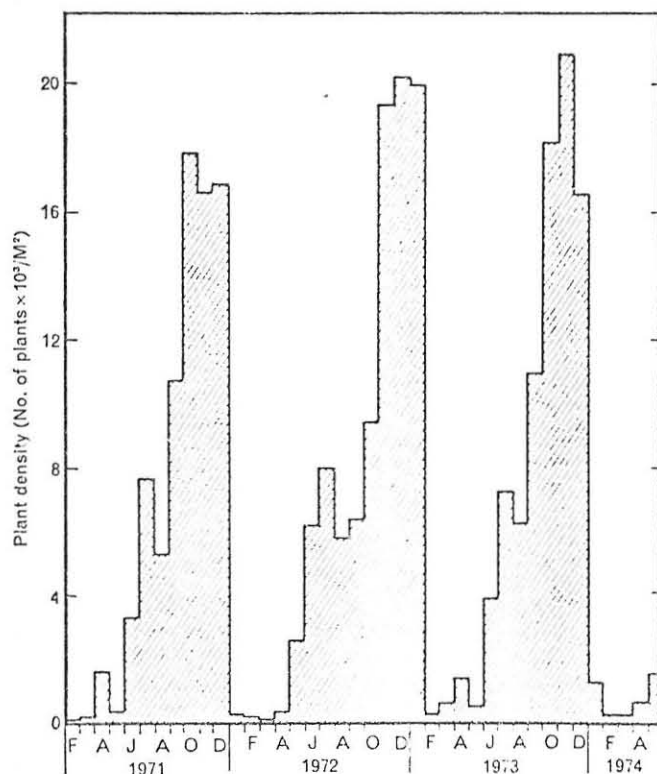


Figure 8 Histogram of average plant density at a riverine site on a tributary stream of the Hendrik Verwoerd Dam.

Nature, however, exercises a certain measure of control and summer rainfall induces an annual flooding of the streams which flushes both the *Azolla* mats and the nutrient-rich waters into the dam. The effect of these waters on the dam is insignificant because of the dilution factor involved. After the floods, *Azolla* plants which have survived in the streams proliferate to reform the original mat conditions [10]. The flooding therefore gives rise to a seasonal fluctuation in the population density of *Azolla* in these streams (figure 8), and the system is self-regulatory. Since these tributary streams are at present economically unimportant, further control of the plant is unnecessary. However, should total eradication be desirable, mechanical or chemical control measures or both would have to be used. This unfortunately poses further problems since there is no selective herbicide for *Azolla* and therefore chemical eradication might produce serious side-effects on other members of the aquatic biota.

#### Conclusions

Growth of *Azolla* is greatly enhanced by the presence of the blue-green alga *Anabaena azollae*, which has formed a close and lasting association with the fern. Currently, there exists a controversy over the ability of the alga to maintain independent growth. The assimilation of nitrogen by the fern from its nitrogen-fixing symbiont is more efficient than the assimilation of nitrogen by the fern from the medium on which it is grown. Nitrogen-

fixing characteristics of *Anabaena azollae* show differences from those of free-living *Anabaena* species, and this may be a consequence of its habitat. Much work remains to be done in elucidating the interdependence of the two organisms.

The appearance of *Azolla* on a water body does not necessarily mean that it will become a problem since the complex growth requirements of the fern may not be satisfied under the particular conditions. However, if the major influences, which include nitrogen fixation, light, pH, nutrient availability, atmospheric gases, turbulence and flooding are favourable, the development of *Azolla* mats is inevitable. In this respect therefore, further research on improved methods of physical and chemical control is necessary.

#### Acknowledgment

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Factors affecting APPENDIX IV  
the growth and development of  
*Azolla filiculoides* Lam

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ABSTRACT

In the Hendrik Verwoerd Dam catchment, the symbiotic aquatic fern, *Azolla filiculoides*, is confined to slow-flowing streams and farm dams. Mat formation causes anaerobic conditions in the underlying waters, promoting the chemical and bacterial liberation of nutrients from the sediments and organic matter. This nutrient flux further stimulates growth of *Azolla* and the consequent proliferation of the mat. Annual floods wash most of the plants into the open lake where they are broken up by wind and wave action. The surviving plants in the streams grow and again form the original mat. Flooding therefore causes a seasonal fluctuation in the population density and the system is self-regulatory.

INTRODUCTION

*Azolla* is a genus of small, heterosporous, aquatic ferns with a wide distribution (Svenson, 1944). Each plant consists of a short, branched, floating rhizome bearing small, alternate, overlapping leaves and has roots which hang down into the water. Colonies of the symbiotic, heterocystous, blue-green alga *Anabaena azollae* Strasb. are found in cavities in the dorsal lobe of each leaf. This alga can fix atmospheric nitrogen and is able to fulfil the nitrogen requirements of the fern. Thus *Azolla* can colonize water bodies which are deficient in nitrogen and unsuitable for the growth of other aquatic plants.

*Azolla filiculoides* Lam., native to south and central America, was introduced into the Colesberg district of South Africa as an aquarium plant some thirty years ago (Ashton and Walmsley, 1976). Due to its excessive growth, the fern was "thrown away" and, due to transport by birds, man and floods, has now spread to several streams, irrigation furrows and farm dams in the area. In these situations, the fern has formed dense mats which block pipelines and cause the development of anaerobic conditions in the underlying waters, rendering these waters unfit for human or animal consumption. It is therefore considered to be a pest. In certain severe cases of infestation, the *Azolla* plants were eradicated by spraying with dieseline (Oosthuizen and Walters, 1961). However, this practice was stopped when animals refused to drink the dieseline-treated water.

With the completion of the Hendrik Verwoerd Dam, the authorities initially feared that like its relative *Salvinia* (Kariba Weed), *Azolla filiculoides* might colonize the open waters of the newly formed dam. Consequently, an autecological study of *A. filiculoides* was initiated in 1971. During the early stages of the study, it was noticed that mats of *Azolla* plants were washed from the streams into the lake by the annual summer floods. These plants were fragmented by wind and wave action and did not survive on the open lake waters. However, residual plants which survived in the streams and small dams, proliferated during early winter and reformed the original mat conditions.

Ashton (1974) showed that the vegetative growth of *A. filiculoides*, grown in a wide range of light and temperature regimes, varied with the pH of the nutrient solution used. Fragmentation of the plants by wind and wave action was also shown to reduce the plant's tolerance of low temperatures and its ability to withstand high light intensities. Ashton and Walmsley (1976) described some of the physico-chemical factors affecting growth and mat formation by *A. filiculoides*, particularly the development of anaerobic, nutrient-rich conditions beneath the mats.

The generalized life cycle of *A. filiculoides* is typical of heterosporous ferns (Sporne, 1962). The continuity of the *Azolla-Anabaena* association is maintained during sexual reproduction (Figure 1) by the inclusion of algal akinetes under the indusium cap of the megasporangium (Ashton and Walmsley, 1976). In the Colesberg district, *A. filiculoides* normally produces sporocarps during the summer, from late September to February, and germinated sporelings of *Azolla* can be found from mid-December to early April. The production of sporocarps is therefore an important

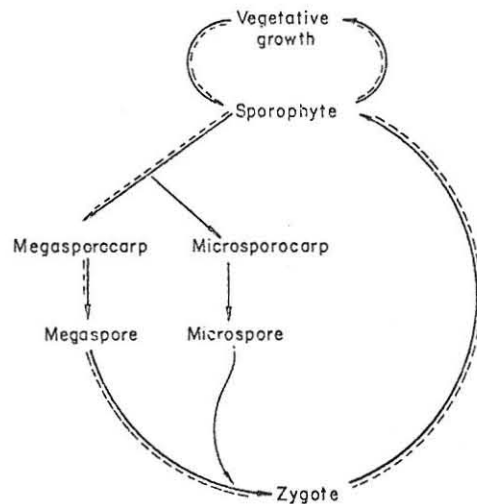


Fig. 1 Life cycle of *Azolla filiculoides* (—) showing the continuation of the association with *Anabaena azollae* (---). [After Ashton and Walmsley, 1976]

means whereby the spread of *A. filiculoides* may be increased. Thus, in order to assess the growth potential of the fern, it is important to understand the mechanisms regulating sporocarp production. This paper presents the results of field and laboratory studies on the factors affecting sporocarp formation in *A. filiculoides* and describes some of the interacting environmental factors which regulate the fern's life cycle in its natural habitat.

#### THE STUDY AREA

A survey carried out during February 1971 to assess the distribution of *A. filiculoides* in the Hendrik Verwoerd Dam catchment area showed that the fern was confined to the area immediately north, south and south west of the lake (Figure 1). *A. filiculoides* was not found in the Orange, Caledon or Kreai Rivers. The fern was found to occur in two habitat types. These were small streams with a continuous inflow and farm dams which received inflows only after periods of rainfall.

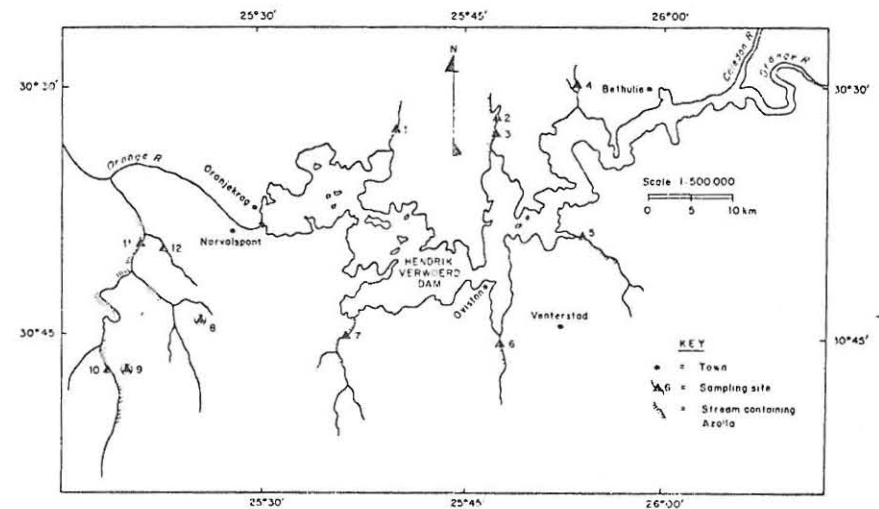


Fig. 2 : Map of study area showing location of sampling sites and distribution of *A. filiculoides*

The study area was located to include all sites containing A. filiculoides in or near the catchment area of the Hendrik Verwoerd Dam. This area is approximately 3000 square kilometres in extent, lying between latitudes 30°28'S to 30°52'S and longitudes 25°18'E and 26°00'E. The generally uniform vegetation of the area is classified as False Upper Karoo (Acocks, 1975), growing on red calcareous clays and solonchic soils (Harmse, 1974), derived from the siltstones, mudstones, limestone and dolerite of the Upper Beaufort Series (Geological Survey, 1970). Within the study area, twelve sampling sites were chosen to cover the whole range of habitats occupied by the fern (Figure 2).

The study area lies within the summer rainfall zone of South Africa and normally receives 300 to 340 mm of rain per year, though exceptional rainfalls of over 900 mm were recorded in 1974 (South African Weather Bureau, 1971-1974). Maximum rainfall is recorded between January and April, usually with an additional short period of high rainfall in October. Rainfall in the study area is mainly of thunderstorm origin, so that large volumes of rain fall in short time intervals. Owing to the flat terrain and rather sparse vegetation cover, sheet erosion occurs in many areas. Flooding and scouring of rivers during the peak rainfall months have a marked effect on riverine aquatic plant life (Ashton, 1974). Maximum and minimum air temperatures vary between 22 °C and -4,9 °C in winter and between 39,8 °C and 22,2 °C in summer, while maximum and minimum water temperatures for the same period varied between 11,2 °C and 3,8 °C in winter and between 29,4 °C and 16,0 °C in summer. Winds which are not seasonal, blow from the north and north west. Maximum light intensities, (measured at midday with a Gossen Trilux Lightmeter), vary from 80 000 Lux in winter to 115 000 Lux in summer.

## MATERIALS AND METHODS

### Routine field measurements

From February 1971 to May 1974, monthly sampling trips were made to the study area. At each sampling site, five sets of pH, oxygen and temperature profiles were measured. These measurements were taken at randomly distributed points within the sampling site and individual

readings were taken from two centimetres below the water surface to the bottom at ten centimetre intervals. pH values were measured with a Metrohm model E444 portable pH meter, while oxygen and temperature readings were taken with a YSI model 54 oxygen meter fitted with a thermistor probe.

### Collection and preparation of samples

A square wooden tube, 100 cm<sup>2</sup> in cross-sectional area and 40 cm in length (Ashton, 1974), was used to obtain ten replicate samples of plants from the Azolla mat at each site for population estimates and chemical analysis. The plants were gently dried and stored on ice in the dark, in numbered plastic bags, for transport to the laboratory. Water samples were collected with a two litre sampling bottle fixed to a pole and similarly stored on ice in the dark during transport to the laboratory. This period of storage was never longer than 36 hours.

In the laboratory, the plants in each sample were counted and the numbers of plants bearing sporocarps noted. The plant material was then dried at 65 °C for three days. Water samples were first filtered through cheese cloth to remove coarse particles of organic material followed by filtration through 0,45 µm Millipore filters and analysed using methods recommended by Colterman and Clymo (1971).

### Laboratory experiments

Large controlled environment rooms were used to give a wide range of photoperiods, light intensities and temperature conditions. Illumination at low light intensities was by means of cool white fluorescent tubes whilst high light intensities were achieved by using a bank of fan-cooled mercury-vapour photo-flood lamps at varying distances. Temperature control within each controlled environment room was within ±0,2 °C. For routine laboratory experiments, A. filiculoides was cultured on a nitrogen-free liquid medium designed to simulate the nutrient conditions occurring beneath an established Azolla mat (Table 1). In experiments where a source of combined nitrogen was required, this was supplied as sodium nitrate to give a nitrogen concentration of 2 mg

TABLE 1 : Composition of culture solution

	Chemical	Concentration (mg l <sup>-1</sup> )
1	MgSO <sub>4</sub> ·7H <sub>2</sub> O	250
2	CaCl <sub>2</sub> ·2H <sub>2</sub> O	200
3	K <sub>2</sub> HPO <sub>4</sub>	20
4	Na <sub>2</sub> HPO <sub>4</sub>	20
(	H <sub>3</sub> BO <sub>3</sub>	2,0
(	MnCl <sub>2</sub> ·4H <sub>2</sub> O	1,5
{5	ZnSO <sub>4</sub> ·7H <sub>2</sub> O	2,5
(	CoCl <sub>2</sub> ·6H <sub>2</sub> O	0,15
(	CuSO <sub>4</sub> ·5H <sub>2</sub> O	0,0015
6	(NH <sub>4</sub> ) <sub>6</sub> Mo <sub>7</sub> O <sub>24</sub> ·4H <sub>2</sub> O	0,005
7	FeNa EDTA	15,0

Concentrations were increased one thousandfold and made up into stock solutions 1 - 7 as shown. These were diluted when making up the culture solution.

l<sup>-1</sup>. When necessary, adjustment of the culture solution pH was accomplished with the use of either 1N HCl or 1N NaOH.

*Azolla filiculoides* plants were acclimatized in large asbestos trays, under the required environmental conditions, for three weeks prior to any experiments. For the analysis of growth rates, weighed samples (2,0 g) of the acclimatized plants were placed in 500 ml capacity plastic dishes containing the appropriate nutrient solution and reweighed five to seven days later. These dishes were painted black to prevent excess light reaching the roots, and to simulate the conditions occurring beneath an *Azolla* mat. A short time interval was used in these growth experiments, since with longer time intervals, the plants became crowded and the growth rate decreased. During both the acclimatization and experimental periods, nutrient solutions were replaced daily by siphoning to avoid disturbance of the plants and to ensure constant nutrient supply. Experiments investigating the effects of environmental factors on sporocarp production were carried out over forty or eighty days, when a

thick mat of plants had developed in the dishes and the lower layers were starting to decompose. Sporocarp production was expressed as the percentage of plants bearing sporocarps.

#### Growth analyses

Relative growth rate, (RGR), defined as the increase of plant material per unit of material initially present per unit of time, was calculated as a mean ( $\overline{RGR}$ ) over the period  $t_2 - t_1$ , from the formula:

$$\overline{RGR} = \frac{\log_e W_2 - \log_e W_1}{(t_2 - t_1)}$$

where  $W_1$  and  $W_2$  are the plant weights at times  $t_1$ , and  $t_2$  (Radford, 1967). Doubling time (in days) was calculated from the formula:

$$\text{doubling time (days)} = \frac{\log_e 2}{(\overline{RGR})} \quad (\text{Mitchell, 1974})$$

In all experiments, ten replicates of each treatment were used to obtain values for the mean and the 95 per cent confidence limits.

#### RESULTS AND DISCUSSION

Field observations showed that mats of *A. filiculoides*, growing in streams which received a continuous inflow, initiated sporocarp production in September, 3 to 4 weeks earlier than mats of *A. filiculoides* growing on farm dams receiving intermittent inflows. These two habitats showed marked differences in pH and nutrient status and were examined in detail. At the same time, laboratory experiments were carried out to assess the effects of photoperiod, light intensity, temperature, pH and nitrogen supply on sporocarp production.

## Laboratory experiments

### (a) Photoperiod

The effects of increasing photoperiod on the relative growth rate and sporocarp production of *A. filiculoides* are shown in Figure 3. With increasing photoperiod, from two hours to fourteen hours, the relative growth rate increased rapidly. With longer photoperiods, the relative growth rate slowly increased to a maximum. However, sporocarp production in *A. filiculoides* was not initiated at photoperiods shorter than eight hours. With increasing photoperiod, the percentage of plants bearing sporocarps increased to a maximum at a photoperiod of fourteen hours. Further increases in photoperiod did not increase sporocarp production. These results

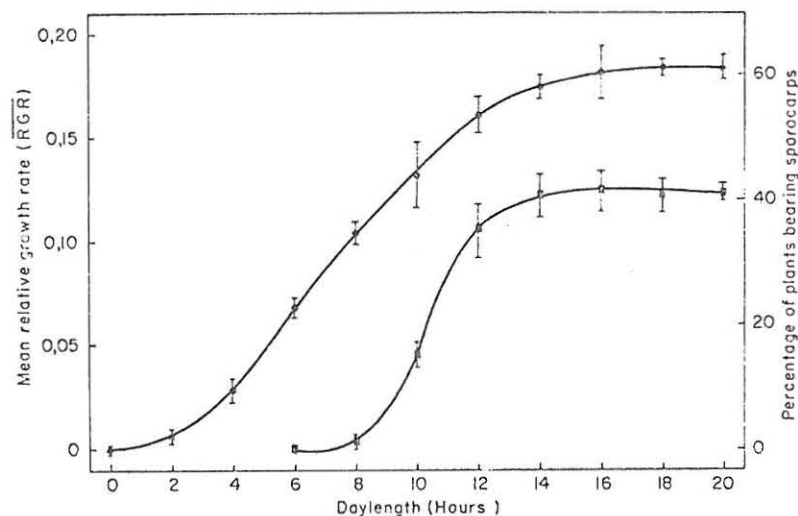


Fig. 3 The mean relative growth rate (RGR) (●—●) and percentage of *A. filiculoides* plants bearing sporocarps (■—■) after forty days growth under different daylength regimes. (Vertical bars indicate 95% confidence limits). [30 000 Lux; pH 9,5; 27,5 °C; nitrogen-enriched medium].

indicate that sporocarp production is dependant on photoperiod and in nature will only take place when daylengths are longer than eight hours, with maximum production occurring during summer when daylengths are longer than twelve hours.

### (b) Light intensity and temperature

Light intensity and temperature were found to have interacting effects on sporocarp production (Figure 4). No sporocarps were produced at temperatures below 17,5 °C and above 35 °C at any of the light intensities used. Within the range 20 °C to 30 °C, the percentage of plants bearing sporocarps increased with increasing light intensity, from 7500 Lux to 30 000 Lux. At 60 000 Lux there was a decrease in the percentage of plants bearing sporocarps over

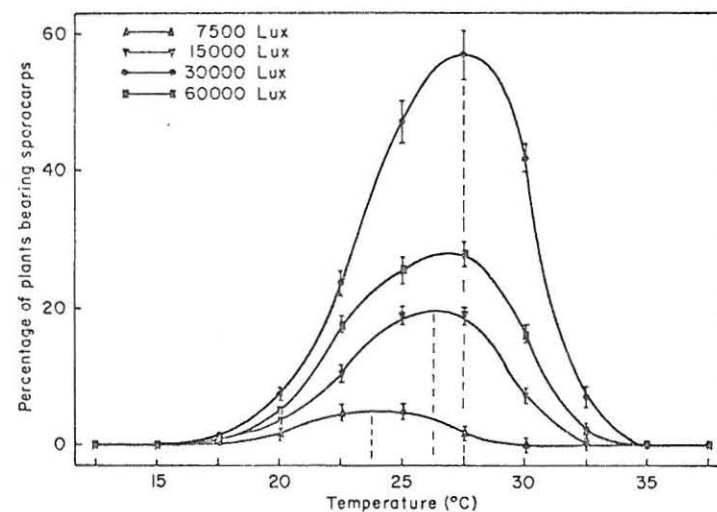


Fig. 4 The effects of temperature and light intensity on the number of *A. filiculoides* plants bearing sporocarps. (Vertical bars indicate 95% confidence limits). [14 hour day; pH 9,5; nitrogen-enriched medium].

the whole temperature range used. An interesting feature of these results is that the 'optimum' temperature for sporocarp production increased with increasing light intensity, to a maximum (27,5 °C) at 30 000 Lux, but a further increase in light intensity to 60 000 Lux caused a slight decrease in 'optimum' temperature. It is evident therefore, that sporocarp formation cannot take place during winter because water temperatures, which often drop below 10 °C, are not favourable. The high 'optimum' temperature also indicates that sporocarp production should only occur during the summer months when water temperatures are high.

During summer, the mean midday light intensity in the study area is approximately 115 000 Lux. Since sporocarp production is inhibited at light intensities greater than 30 000 Lux in the laboratory, this indicates that plants of *A. filiculoides* in the lower layers of the mat, shaded from direct sunlight by the overlying layers, are best able to produce sporocarps. This has been confirmed by careful examination of thick mats of *A. filiculoides* in the field. The plants in the upper two to three layers did not produce sporocarps, whilst in the lower layers up to 85 per cent of the plants had produced sporocarps.

(c) Temperature and pH

The effects of pH on the sporocarp production of *A. filiculoides* plants grown at three different temperatures are shown in Figure 5. At the 'optimum' temperature of 27 °C, sporocarp production was greatest in the pH range 8,5 to 10,0 with a maximum at pH 9,5. Sporocarp production was minimal at pH values below 8,5 or above 10,0. At temperatures above 27 °C or below 22 °C, sporocarp production was greatly reduced over the whole pH range. These results show that at the water temperatures of 20 °C to 23 °C (which are recorded during summer), sporocarp formation is optimal when the pH of the water is between 9,5 and 10,0.

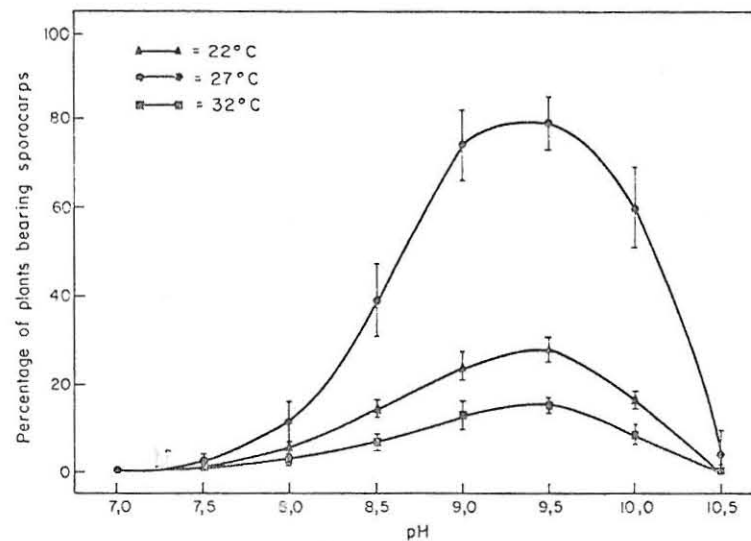


Fig. 5 The influence of pH and temperature on the number of *Azolla* plants bearing sporocarps. (Vertical bars indicate 95% confidence limits). [30 000 Lux; 14 hour day; nitrogen-enriched medium].

(d) Nitrogen source

Previous work by Ashton (1974) has shown that as plant density per unit area increased during mat formation, the rate of fixation of atmospheric nitrogen decreased to a very low level. However, even under favourable environmental conditions in the laboratory, *A. filiculoides* only formed sporocarps when the plants were in an established mat and the rate of vegetative growth has decreased to a minimum (Table 2). The nitrogen requirements of the fern must therefore be partly fulfilled by the uptake of a source of combined nitrogen from the underlying waters. The effects of nitrate concentration on sporocarp production were therefore investigated. As can be seen in Figure 6, at the three temperatures used, sporocarp production increased with increasing nitrate concentration to a maximum at 1 mg l<sup>-1</sup> NO<sub>3</sub>-N. A nitrate concentration of 10 mg l<sup>-1</sup>

TABLE 2 : The effect of increasing plant density on the mean relative growth rate (RGR), doubling time and percentage of *Azolla* plants bearing sporocarps. (30 000 Lux; 14-hour day; 27,5 °C; pH 9,5; nitrogen-enriched medium).

Time elapsed (days-	RGR (% day <sup>-1</sup> )	Plant density (no. of plants dm <sup>-2</sup> )	Doubling time (days)	% of plants with sporocarps
0	20,30	3,18	3,42	0
5	20,30	3,78	3,42	0
10	20,36	24,31	3,39	0
15	16,51	55,49	4,20	0
20	6,72	77,64	10,35	0
25	2,89	89,73	23,90	2,8
30	1,21	95,33	57,76	9,7
40	0,89	104,24	77,02	29,1
60	0,61	117,86	115,52	43,4
80	0,32	125,75	231,05	47,2

inhibited sporocarp production. At 27 °C, sporocarp production was maximal, while at both 22 °C and 32 °C, sporocarp production was reduced.

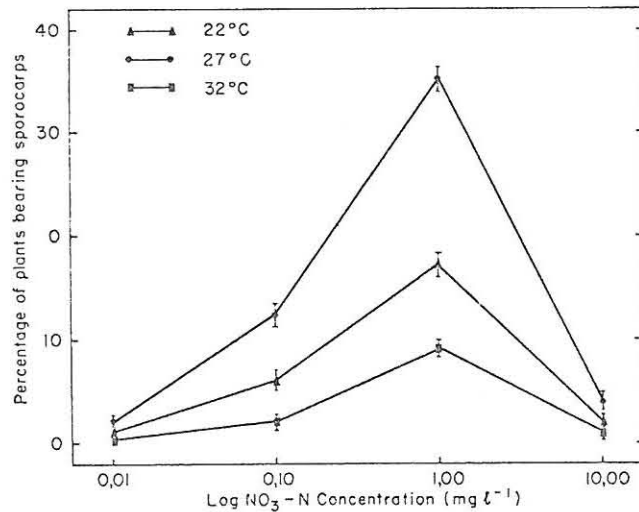


Fig. 6 The effects of nitrate concentration and temperature on the number of *Azolla* plants bearing sporocarps. (Vertical bars indicate 95% confidence limits). [30 000 Lux; 14-hour day; pH 9,5].

### Field studies

In many of the streams in the study area, deep pools are formed by flood-scouring of soft shales and mudstones behind dolerite and limestone ridges which lie at right-angles to the direction of stream flow. After the annual summer flooding, the flow-rate in these streams is reduced to a low but continuous level. The pH of the water varies between 8,2 and 9,4, with high levels of calcium, magnesium, sulphate and carbonate and very low levels of nitrogen and phosphorus. When a mat of *A. filiculoides* develops over a pool, the plants in the lower layers of the mats are often in a state of decomposition and a thick layer of sedimented plant material develops on the floor of the pool. Bacterial and chemical degradation of these organic sediments causes the development of anaerobic conditions and a consequent lowering of the pH of the water. The levels of nutrients, particularly phosphorus, nitrogen, iron and manganese, are increased due to their liberation from these sediments.

The continuous inflow of nutrient-poor high pH water immediately below the mat became partly mixed with the nutrient-rich lower water and caused the development of intense pH stratification (Figure 7). From June 1972

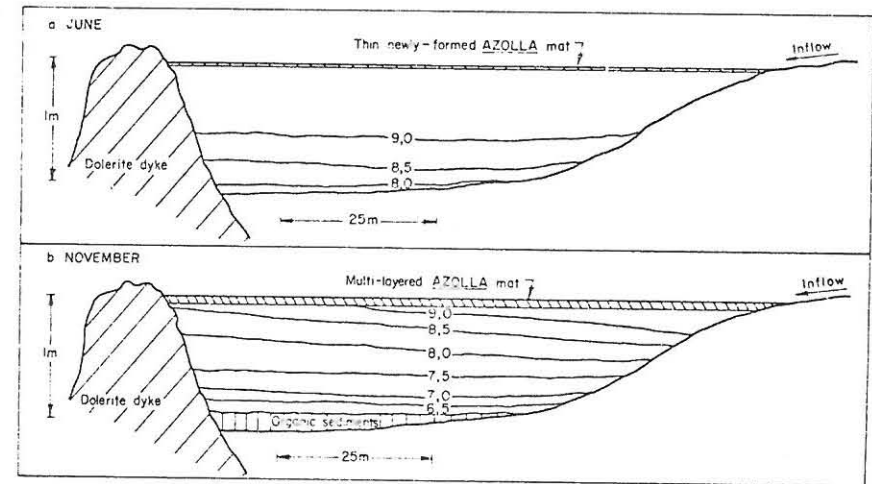


Fig. 7 The development of intense pH stratification beneath a developing *Azolla* mat, from (a) June 1972 to (b) November 1972, at a riverine station receiving a continuous inflow. (Horizontal scale greatly reduced).

to November 1972, the concentrations of  $\text{PO}_4\text{-P}$  and  $\text{NO}_3\text{-N}$ , at a depth of 5 cm below the *Azolla* mat at the riverine station, increased from approximately  $10 \mu\text{g l}^{-1}$  to  $650 \mu\text{g l}^{-1}$  and  $35 \mu\text{g l}^{-1}$  to  $1500 \mu\text{g l}^{-1}$  respectively. Thus the partial mixing of the nutrient-poor high pH inflow with the nutrient-rich lower waters ensured that sufficient nutrients, particularly nitrate and phosphate, were available for continued proliferation of the mat and sporocarp development, while the pH of the surface water was maintained at a high optimum level. In this situation, although the conditions of water pH and nitrate concentration were favourable for sporocarp production from early spring, maximum sporocarp production only took place in early early summer when the conditions of temperature and photoperiod were favourable.

In the case of *A. filiculoides* plants growing in a small farm dam which received intermittent inflows, the situation was somewhat different. As in the case of the riverine station, bacterial and chemical degradation of sedimented plant material below an *Azolla* mat caused anaerobic conditions to develop with a decrease in the pH level of the water (Figure 8). The levels of phosphorus, nitrogen, iron and manganese released from these sediments also increased markedly. However, since there was no inflow of high pH water, pH stratification did not develop to the same extent as the riverine station. The pH of the surface waters was therefore lowered. Evapotranspiration from the *Azolla* mat, (measured as  $420 \text{ ml m}^{-2} \text{ day}^{-1}$  in the laboratory, at 60 000 Lux and 27 °C), caused a lowering of the water level and further increased the concentrations of available nutrients.

From June 1972 to November 1972, the concentrations of  $\text{PO}_4\text{-P}$  and  $\text{NO}_3\text{-N}$  at a depth of 5 cm below the *Azolla* mat at the farm dam station increased from approximately  $12 \mu\text{g l}^{-1}$  to  $3900 \mu\text{g l}^{-1}$  and  $30 \mu\text{g l}^{-1}$  to  $9200 \mu\text{g l}^{-1}$  respectively. Despite being approximately six times higher than those recorded in the riverine station, these high nutrient levels caused only a slight increase in the growth of *A. filiculoides*. However, due to the low pH level and high nitrate concentration of the surface water, sporocarp formation was inhibited. It therefore appeared that sporocarp formation would only be initiated when the dam received inflows of nutrient-poor, high pH water following rainfall.

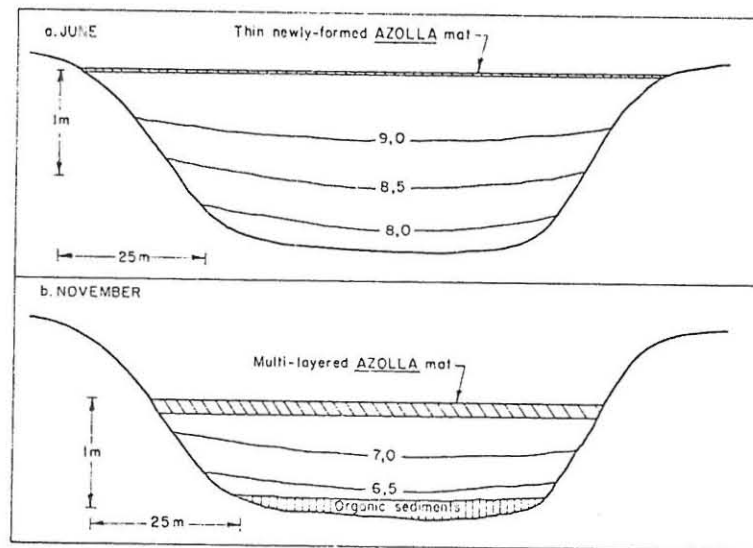


Fig. 8 The decrease in pH caused by anaerobic conditions beneath an *Azolla* mat developing on a farm dam receiving no inflow during the period (a) June 1972 to (b) November 1972. (Horizontal scale greatly reduced).

When the monthly values for surface water temperature, pH and percentage of *Azolla* plants bearing sporocarps in the two habitat types were compared with the mean monthly rainfall records for the study period (Figure 9), the differences between the two habitat types were clearly defined. In the riverine station receiving a continuous inflow of high pH water, the pH values remained in the optimum range and maximum sporocarp production was only recorded when the water temperature rose above 20 °C. On the other hand, in the farm dam station which only received inflows after rainfall, the surface pH values decreased to a level unfavourable for sporocarp formation with increasing mat development. Despite the rise in temperature to an optimum level in September, sporocarp formation was dependant on the inflow of high pH runoff following rains in October. This increase in the surface pH value was of short duration, but was enough to trigger off sporocarp formation, despite low rainfalls in November. With further rainfall in December and January, Sporocarp formation rose to a maximum.

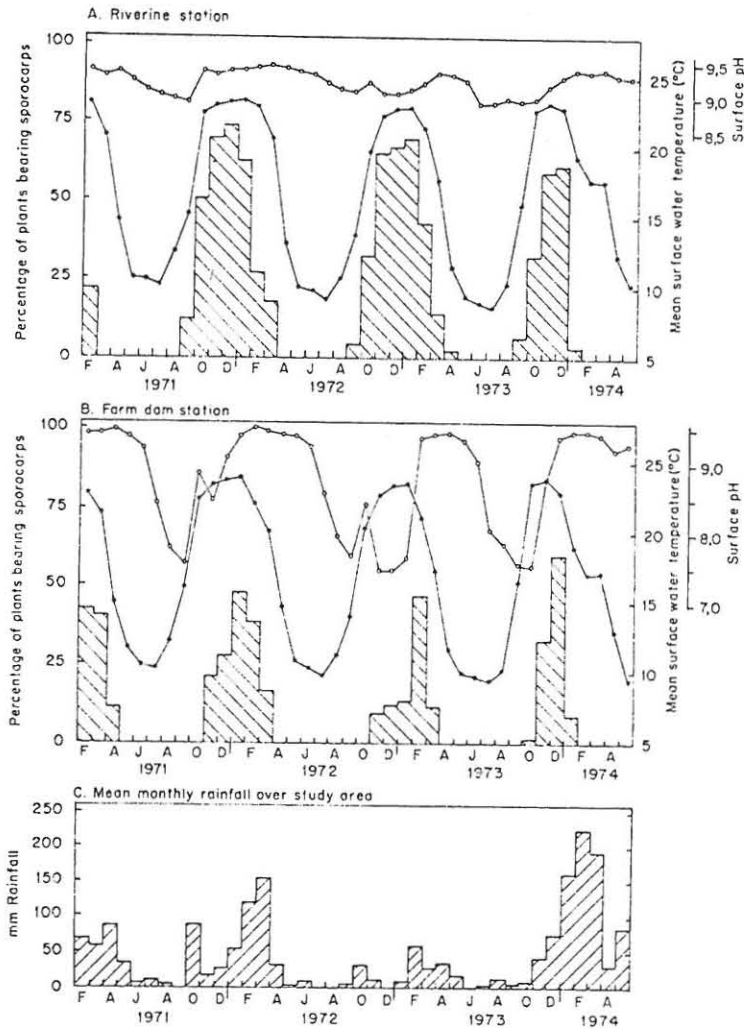


Fig. 9 A comparison of the influence of mean surface water temperature (—●—) and pH (—○—) on sporocarp production by *Azolla* plants at (A) a riverine station with continuous inflow and (B) a farm dam with intermittent inflow from February 1971 to May 1974. [Figure C shows the mean monthly rainfall for the study area during this period].

In both the riverine and farm dam stations, heavy rainfalls from January to March caused flooding, with a consequent dispersal of the *Azolla* plants and sporocarps. *Azolla* plants are extremely fragile and easily fragmented. Previous work has shown that fragments of *A. filiculoides* plants cannot tolerate high light intensities and soon die (Ashton, 1974). The sporocarps on the other hand do not fragment easily and thus serve as a source of infestation for localities further downstream.

After the annual floods, surviving plants and sporocarps of *A. filiculoides* proliferated to reform the original mat conditions in the streams and dams. The flooding therefore gave rise to a seasonal fluctuation in the population density of *A. filiculoides*, (see Figure 10 for results from the riverine station), and the system may be considered self-regulatory. Since the growth of *A. filiculoides* on the streams and farm dams in the area poses no economic problems at present, further control is unnecessary.

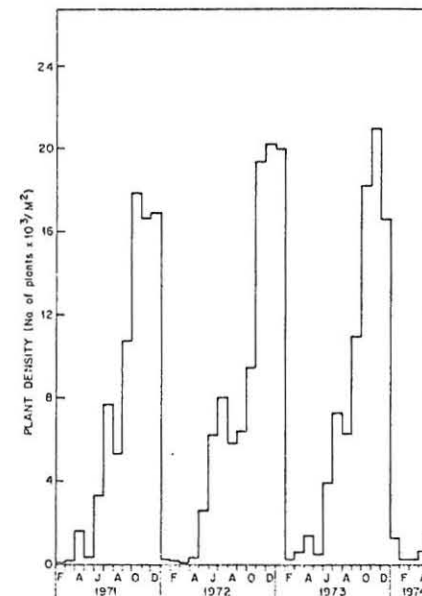


Fig. 10 Histogram of average plant density at a riverine station (After Ashton, 1974)

## CONCLUSIONS

1. Sporocarp formation in Azolla filiculoides only occurs in established mats and is regulated by the interacting effects of photoperiod, light intensity, temperature, pH and nitrogen supply.
2. In a riverine station receiving a continuous inflow, sporocarp production is regulated by water temperature and photoperiod. However in a farm dam station receiving intermittent inflows, photoperiod and surface pH values regulate sporocarp production.
3. After dispersal by annual summer floods, sporocarps of A. filiculoides are an important source of infestation in new localities.
4. Flooding gives rise to seasonal fluctuations in the population density of A. filiculoides plants and the system is self-regulatory.

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