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A COMPARATIVE STUDY OF AZOLLA IN THE NETHERLANDS

A. H. PIETERSE*, L. DE LANGE** and J. P. VAN VLIET**

* Koninklijk Instituut voor de Tropen, Afdeling Agrarisch Onderzoek, Amsterdam
** Hugo de Vries Laboratorium, Universiteit van Amsterdam

SUMMARY

Fifteen populations of *Azolla* were sampled in the western and north-eastern parts of The Netherlands. After cultivation under identical conditions in a greenhouse they could be divided into two species which had previously been recorded from The Netherlands under the names of *Azolla filiculoides* Lam. and *Azolla caroliniana* Willd. Populations which appeared to be morphologically intermediate between these two species were eventually found to be true *A. filiculoides*. Although identification of certain forms in the field remains difficult, the two species can always be distinguished under a microscope by the number of cells forming the trichomes on the surface of the upper lobe of the leaflets, the septation of the glochidia and the surface structure of the megaspores. Other characteristics which have been reported in the literature are not always consistent. However, well-developed specimens of *A. filiculoides* can easily be recognised from their general appearance. In the autumn, when the plants become reddish as a result of anthocyanin production, *A. filiculoides* turns to a purer red than the plants of the other species which become more browny red. In both species the anthocyanins contain the relatively rare anthocyanidins luteolinidin and apigenidin. Anthocyanin production was found to be influenced by temperature and water composition but not by day-length. Well-developed forms of *A. filiculoides* generally occur in more eutrophic water than plants of the other species. According to the revised classification of the New World species of *Azolla* by SVENSON (1944), the Dutch specimens previously referred to as *A. caroliniana* do not belong to this species, but to either *A. mexicana* Presl or *A. microphylla* Kaulfuss.

1. INTRODUCTION

In the second half of the nineteenth century American specimens of the genus *Azolla*, small, free-floating water-ferns, were introduced into botanical gardens in Western Europe and soon escaped into nearby waters. Consequently, these plants became permanently integrated in the flora of several European countries. The introduced plants were identified as *Azolla caroliniana* Willd. and *Azolla filiculoides* Lam., based mainly on the classification of STRASBURGER (1873). This author, following the earlier classification of METTENIUS (1867), considered only these two *Azolla* species as being indigenous to the American continent. All additional taxa previously recorded as occurring there were regarded by him as synonymous with one of these two species. In 1944 SVENSON, however, classified the New World representatives of *Azolla* into four species and re-instated *A. mexicana* Presl and *A. microphylla* Kaulfuss.

In The Netherlands, plants identified as *A. caroliniana* were introduced about 1880 into the botanical garden of the University of Leiden (VAN EEDEN

1915; WAAGE 1929). It had already infested large areas in the western part of the country when around 1895 *A. filiculoides* was discovered for the first time in the Dutch waterways (BERNARD 1904; HEIMANS 1915a, b). At present the first species occurs mainly (perhaps exclusively) in the north-eastern part of the country, in the provinces of Overijssel and Friesland. *A. filiculoides* is very common in the western provinces, but also occurs in the middle and north-eastern regions. HEIMANS (1915a, b) and VAN OOSTSTROOM (1948) suggested that *A. filiculoides* crowded out its smaller relative in the western part of The Netherlands, possibly because in nature the latter species seldom forms spores in this country. According to SEGAL (1966), there is a marked difference between the habitats of the two species in The Netherlands: *A. filiculoides* prefers eutrophic or slightly brackish water while its smaller relative grows in moderately eutrophic water. In this context it was suggested by Segal that *A. caroliniana* has disappeared from the western part of the country as a result of water pollution. On the other hand HEIMANS (1915b) states that an intentional introduction of this species into the – at that time presumably still oligotrophic – waters in the eastern part of the country proved unsuccessful.

Although BERNARD (1904) described the two species in The Netherlands and confirmed the essential morphological differences mentioned by STRASBURGER (1873), it is not always easy to identify vegetative material in the field, especially as *Azolla* plants may vary considerably under the influence of external conditions. In many cases, forms have been observed which are more or less intermediate between the typically curly, partly emerged and larger plants of *A. filiculoides*, and the smaller, isodiametric and more flattened plants of the other species. In this context it may be expected that various reports on the occurrence of *Azolla* in The Netherlands are not always correct as far as the specific name is concerned.

The revised classification of SVENSON (1944) – raising the question of the specific identification of the Dutch material – and reports of the occurrence of intermediate forms prompted a re-investigation of *Azolla* in The Netherlands. This study was not restricted to morphological aspects only but included the influence of the environment on morphological characteristics as well.

2. MATERIALS AND METHODS

During the period October–November 1975, 15 population samples of *Azolla* were gathered from ditches and canals in the provinces of Friesland, Overijssel, Utrecht and North-Holland. On the day of collection 9 of them were identified as *Azolla filiculoides* Lam., 3 as *Azolla caroliniana* Willd. and 3 were considered to be intermediate forms. The initial identification was based only on the vegetative characteristics mentioned by VAN OOSTSTROOM (1948) as sporocarps were lacking. For a comparison a sample collected in May 1976 in Venezuela and identified as *A. caroliniana* was also included in the experiments. Data on the provenance of the populations are presented in *table 1*.

Table 1. Details of origin, identification on day of collection and morphological data after cultivation under identical conditions in a greenhouse, of the various *Azolla* samples.

Sample no.	Provenance		Date	Indentification on day of collection	Sporocarps after cultivation	Septation of glochidia	N. of cells of trichomes
	Locality	Province					
1	Joure	Friesland	10.11.75	intermediate f.	+	—	1
2	Wolvega	Friesland	10.11.75	A. caroliniana	+	+	2
3	Oldemarkt (Hogeweg NE-side)	Overijssel	15.10.75	A. caroliniana	+	+	2
4	Oldemarkt (Hogeweg SW-side)	Overijssel	15.10.75	A. caroliniana	+	+	2
5	Maarssen (Molenpolder E-side)	Utrecht	17.11.75	intermediate f.	+	—	1
6	Maarssen (Molenpolder W-side)	Utrecht	17.11.75	A. filiculoides	+	—	1
7	Oud Maarsseveen	Utrecht	17.11.75	intermediate f.	+	—	1
8	Nieuw Maarsseveen	Utrecht	17.11.75	A. filiculoides	+	—	1
9	Weesp	N. Holland	24.11.75	A. filiculoides	+	—	1
10	Ouder-Amstel (Polder Rondehoep)	N. Holland	16.10.75	A. filiculoides	+	—	1
11	Zuiderwoude	N. Holland	22.10.75	A. filiculoides	+	—	1
12	Oostzaan	N. Holland	22.10.75	A. filiculoides (small f.)	+	—	1
13	Heemskerk	N. Holland	22.10.75	A. filiculoides	+	—	1
14	Middenbeemster	N. Holland	22.10.75	A. filiculoides	+	—	1
15	Schermer	N. Holland	22.10.75	A. filiculoides	+	—	1
16	Venezuela (Lake Taiguaiguay)	—	27.05.76	A. caroliniana	—		2

The population samples were cultured separately in an unheated, but frost-free greenhouse in polyethylene trays (45 × 30 × 8 cm) which contained a 5 cm layer of wet pot soil. During the summer season (May–August) of 1976 all samples formed micro- and macrosporocarps with the exception of the one from Venezuela.

The following investigations were carried out:

a. comparative biometric studies of the general morphology;

b. examination of colour and pigments with the aid of standard colour charts (Munsell Color Company, 1963) and paper- and thin-layer chromatography; for the latter investigation fresh leaves were extracted in methanol/HCl 0.1%; the extract was concentrated in vacuum and partitioned against petroleum ether to remove the chlorophyll pigments; the red and yellow pigments were purified by paperchromatography in BAW and formic acid/HCl, and subsequently identified chromatographically and spectrophotometrically (HARBORNE 1967), luteolinidin from the flowers (corollas) of *Reichsteineria cardinalis* (Gesneriaceae) being used as reference material;

c. a light-microscopic examination of the trichomes on the upper lobes of the leaflets in microtome sections of 5 µm (embedding in glycol-methacrylate and staining with P.A.S., according to FEDER & O'BRIEN 1968), as well as an examination of the leaf surface and the trichomes with a Scanning Electron Microscope (SEM) Cambridge Stereoscan II, without any previous treatment of the wet plant material;

d. a light-microscopic study of the structure of the glochidia;

e. a SEM examination of the surface structure of the gold-coated macrospores;

f. reciprocal translocation experiments in isolation nets with samples 3 and 10 according to DE LANGE (1974) during the period October 1975–October 1976; *A. cf. caroliniana* specimens of sample 3 from Oldemarkt were placed in a net at Ouder-Amstel and *A. filiculoides* specimens of sample 10 from Ouder-Amstel were placed in a net at Oldemarkt, adding an isolation net with a sample of the local population at each site as a blank;

g. studies in the laboratory on the effect of temperature, day-length, medium composition and the presence of the symbiont *Anabaena azollae* Strasburger on anthocyanin formation in *A. filiculoides* as well as in the other species; the plants were cultured in 1000 ml beakers containing about 500 ml of the nutrient medium under an illumination from cool-white fluorescent tubes of 12,000 erg cm^{-2} s^{-1} intensity; the temperature was regulated by placing the beakers in a water bath with circulation and temperature conditioning type Grant Cambridge Ltd. 55 B 4; two different media were utilized viz. an artificial medium containing, in mg/l: P (PO_4^{3-}) 0.5, N (NO_3^-) 1.12, HCO_3^- 67, SO_4^{2-} 36, Cl^- 87, Ca^{2+} 49, Mg^{2+} 9, Na^+ 23, K^+ 3.8, Fe^{3+} 1, supplemented with traces of Zn, Mn, B, Cu, Co and Mo, and natural ditch water which contained, as far as analyzed: P (PO_4^{3-}) 0.064, P-tot. 0.14, N (NO_3^-) tr., N (NO_2^-) 0.49, N (NH_4^+) 9.1, SO_4^{2-} 140, Cl^- 310, Ca^{2+} 80, Mg^{2+} 63, Na^+ 232, K^+ 19 mg/l; the pH of the artificial medium was 4.8, the pH of the ditch water 8.5; the

photoperiods consisted of 16 hs of light and 8 hs of darkness and 8 hs of light and 16 hs of darkness on a 24 hs schedule; *Anabaena* free plants were obtained by NaClO treatment.

h. chemical analyses of water from the different sites were carried out at the Hugo de Vries Laboratory, according to the standard methods applied there and published by DE LANGE & DE RUITER (1977).

3. RESULTS

3.1. Morphology

When cultivated in the greenhouse the samples were in a poor condition during the winter season – some *A. filiculoides* samples showing characteristics of the "intermediate" forms – but grew vigorously in the subsequent spring and summer. Luxuriously growing plants of *A. filiculoides* became clearly discernible as such by their curly foliage, larger size and wider hyaline margins (consisting of 3–4 rows of cells, as against 1–2 rows in the smaller species). The length of the upper leaf lobes of such forms of *A. filiculoides* varied from 1.5 mm to 2.0 mm, as against 0.7 mm to 0.9 mm in the other species. However, crowded cultures of the smaller species also had a curly appearance although less pronounced.

The relatively small "intermediate" forms (samples 1, 5 and 7) had flat fronds. Their upper leaf lobes varied in length from 0.7 mm to 0.9 mm, with not very distinct hyaline margins. After prolonged cultivation, however, they were no longer distinguishable from the other samples of *A. filiculoides*.

No consistent differences in shape of the upper lobes of the leaflets of the two species could be ascertained.

Sample 12 from Oostzaan remained slightly smaller than the other samples of *A. filiculoides* during an extended period of time (8 months) and was the first to produce sporocarps in the spring. Eventually, however, it became indistinguishable from the other samples of this species. In fact, after prolonged cultivation under identical conditions in the greenhouse, no differences were observed amongst the 12 samples of this species, or amongst the cultivated Dutch samples of the other species. In *fig. 1* typical forms of both species are shown in a mixed culture.

The sample from Venezuela did not form sporocarps. Its general morphology after cultivation in the greenhouse was more or less intermediate between the two species from The Netherlands. The plants were larger and curlier than the Dutch specimens of the smaller species, but always remained distinctly smaller than luxuriant specimens of *A. filiculoides*.

3.2. Pigments

As regards the overall colour, it appeared that the plants identified as *A. caroliniana* were darker green than those of *A. filiculoides* which were more glaucous. The results of a comparison with Munsell Color Charts (1963) are given in *table 2*.

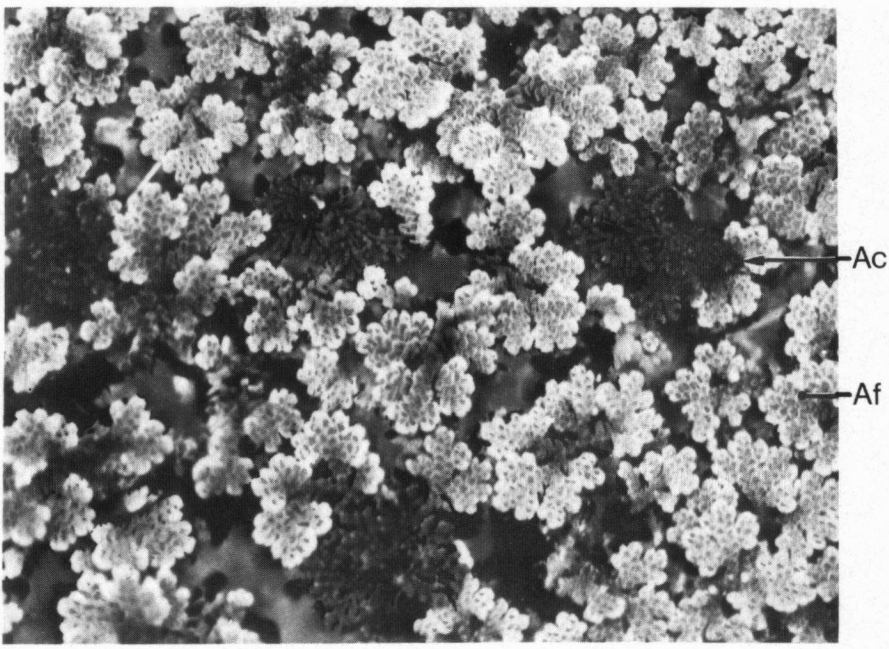

Fig. 1. Typical forms of the two Dutch *Azolla* species in a mixed population after cultivation under identical conditions in a greenhouse (A.f. = *A. filiculoides* Lam., A.c. = *A. cf. caroliniana* Willd.).

A more pronounced colour difference became obvious in the autumn, when *A. filiculoides* plants turned a purer red, and plants of the other species turned a more browny red. This change initiated from the margins of the upper lobes of the leaflets.

By chromatographic analyses of the anthocyanidins two pigments could be identified in both species, *viz.*,

pigment 1: $\max^{(MeOH + HCl\ 0.1\%)} = 494$ nm, in UV 277 nm.
$\max^{(MeOH + AlCl_3)} = 542$ nm.
Rf (after hydrolysis) in TLC cellulose Forestal = 0.58.

pigment 2: $\max^{(MeOH + HCl\ 0.1\%)} = 478$ nm, in UV 275 nm.
$\max^{(MeOH + AlCl_3)} = 478$ nm.
Rf (after hydrolysis) in TLC cellulose Forestal = 0.77.

The characteristics of pigment 1 – which was the main pigment – agree with published data of a luteolinidin glycoside, those of pigment 2 of an apigenidin glycoside (HARBORNE 1967).

Table 2. Colour codes, according to Munsell Color Chart (1963), of the various *Azolla* samples after cultivation under identical conditions in a greenhouse.

Sample no.	No. and code of hue	Value of lightness	Degree of saturation	Sample no.	No. and code of hue	Value of lightness	Degree of saturation
1	7.5 GY	5	6	9	7.5 GY	4	6
2	5 GY	7	8	10	7.5 GY	5	6
3	5 GY	6	8	11	7.5 GY	5	6
4	5 GY	5	6	12	7.5 GY	5	6
5	7.5 GY	4	6	13	7.5 GY	5	6
6	–	–	–	14	–	–	–
7	7.5 GY	4	6	15	7.5 GY	5	4
8	7.5 GY	6	6	16	–	–	–

Table 3. Chemical composition of the water at the translocation sites.

	pH	Conduct.	Alkal.	Cl^-	SO_4^{2-}	$P.PO_4^{3-}$	P-tot.	$N.NO_3^-$	$N.NO_2^-$	$N.NH_4^+$	Ca^{2+}	Mg^{2+}	Na^+	K^+	TOC
		K 25	meq/l						mg/l						
Ouder-Amstel	7.2	380 × 10	7.1	1000	62	1.0	1.2	0.1	0.081	0.02	140	69	540	31	39
Oldemarkt	7.1	96 × 10	2.6	175	58	0.026	0.050	trace	0.067	0.37	49	17	97	7	16

3.3. Trichomes of the upper lobes of the leaflets

The trichomes on the surface of the upper lobes of the leaflets were observed to be unicellular in *A. filiculoides* and bicellular in the other species (including the sample from Venezuela; see *table 1*). Cross-sections of upper lobes of the leaflets are shown in *fig. 2a* and *2c*, and SEM micrographs of the surface of upper lobes in *fig. 2b* and *2d*. In both figures it is clearly noticeable that in *A. filiculoides* trichomes are formed by protuberances of single epidermal cells, whereas in the other species the trichomes consist of a relatively less protruding epidermal cell bearing a second cell on top.

3.4. Glochidia

The glochidia of *A. filiculoides* plants are non-septate, whereas those of the smaller species are septate at regular intervals *(table 1)*.

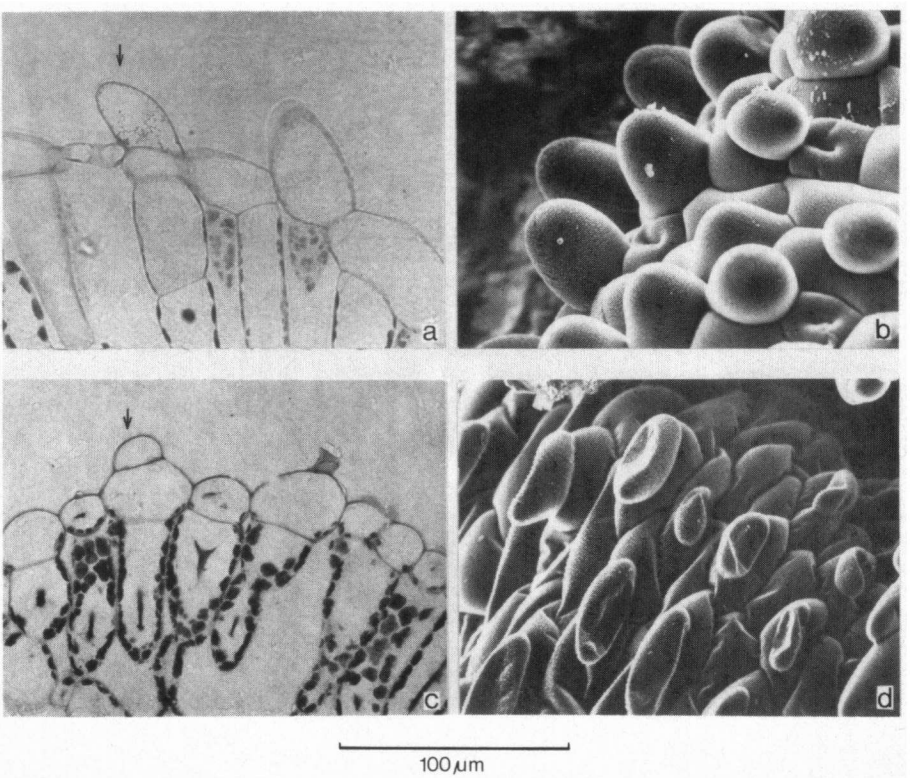

Fig. 2. a. Cross-section of the upper lobe of a leaflet of *A. filiculoides*. b. Scanning electron micrograph of the surface of the upper lobe of a leaflet of *A. filiculoides*. c. Cross-section of the upper lobe of a leaflet of *A. cf. caroliniana*. d. Scanning electron micrograph of the upper lobe of a leaflet of *A. cf. caroliniana*.
Note the unicellular trichomes in *A. filiculoides* and the bicellular trichomes in *A. cf. caroliniana*.

3.5. Macrospore wall

SEM micrographs of the surface structure of the macrospores are reproduced in *fig. 3*. In *A. filiculoides* this surface is characterized by dispersed clusters of thin hairs, which appear to be situated above protuberances appr. 30 μm in width. At least some of these hairs seem to originate from within these protuberances. On the other hand, in the second species a more evenly spread mass of hairs is discernible as well as small papillae of appr. 3–4 μm width emerging between the hairs. In *A. filiculoides* an equatorial girdle around the macrospore apparatus is clearly visible, to which hairs are attached, whereas in the other species no such girdle could be observed.

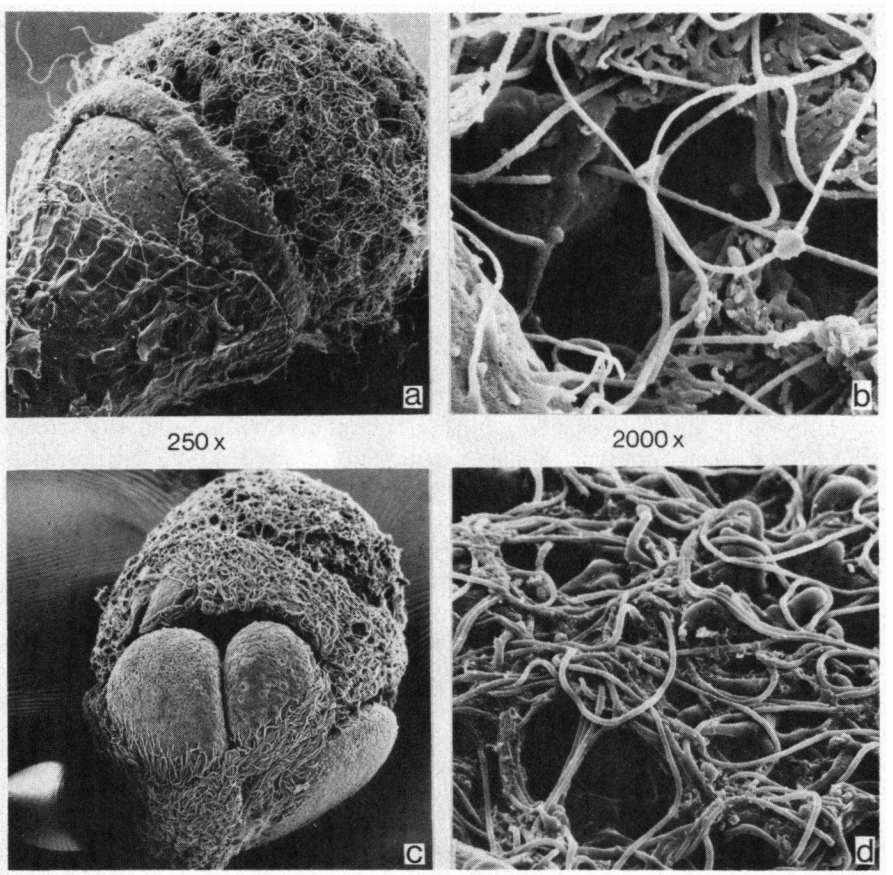

Fig. 3. a. Scanning electron micrograph of a macrospore apparatus of *A. filiculoides*. b. Detail of a., showing the lower edge of the equatorial girdle and the perisporal hairs which are attached to protuberances of the macrospore wall. c. Scanning electron micrograph of a macrospore apparatus of *A. cf. caroliniana*. d. Section of c., showing the perisporal hairs covering the surface of the macrospore. Note the protuberances of the macrospore wall among the hairs.

3.6. Translocation experiments

In Oldemarkt in the province of Overijssel the *A. filiculoides* plants clearly increased during the autumn of 1975 while the colour changed from green to reddish. During the winter season the red plants remained alive in the net, even after a frost period. In March all plants had disappeared, but they returned in May, probably from vegetative fragments in the mud at the bottom of the net, as sporocarps had not been observed in the preceding autumn. In June the net was filled with vigorously growing plants which remained alive up to the autumn of 1976 when the experiment was terminated. In the net at the same site with *A. cf. caroliniana* specimens of local provenance a similar development was observed, *i.e.*, up to March floating plants were present, which later seemed to disappear but which returned to the surface of the water in May–June. On the other hand, in Ouder-Amstel in the province of North-Holland growth of *A. cf. caroliniana* was poor, the plants completely disappearing in the spring. Although the local *A. filiculoides* plants in the other isolation net clearly multiplied during the autumn, they also disappeared in the following spring and summer season. The original *A. filiculoides* vegetation at this site vanished too and even at the end of the summer no floating specimens were found in this ditch. Chemical water analyses of the two sites *(table 3)* indicated a marked difference, though both waters belong to the eutrophic type.

3.7. Conditions affecting anthocyanin synthesis

In separate laboratory tests indications were found that anthocyanin synthesis is furthered by low temperatures (5°–10°C), as well as by natural ditch water as compared with the artificial culturing medium. Differences in day length (16 hs and 8 hs) did not seem to have an influence in this respect. The presence of *Anabaena* did not seem to be essential for the process.

3.8. Chemical analyses of water from the different sites

The results of the chemical analyses of the water *(table 4)* show that well-developed forms of *A. filiculoides* occur in more eutrophic water as compared to "intermediate" forms or plants of the other species. The water at Oostzaan, where the plants of *A. filiculoides* were clearly smaller in size, had an ionic composition markedly below the average found for populations of this species, but higher than that of the "intermediate" form.

4. DISCUSSION

After cultivation under identical circumstances the 16 samples could be divided into the two American species which were delimited by METTENIUS (1867) and STRASBURGER (1873) under the names of *A. filiculoides* Lam. and *A. caroliniana* Willd. The forms which seemed to be morphologically intermediate proved to be modifications of *A. filiculoides*, thus illustrating the great variability of this species. This is emphasized by the fact that the upper lobes of the leaflets of

the sample from Oostzaan, which was eventually indistinguishable from the other samples of *A. filiculoides*, remained slightly smaller during the first eight months of growing under identical circumstances in the greenhouse. In all probability, genotypes of *Azolla* which morphologically differ from the two species discussed do not occur in The Netherlands.

Vegetative material of the two species can always be distinguished by the structure of the trichomes on the surface of the upper lobes of the leaflets, which are unicellular in *A. filiculoides* and bicellular in the other species. This is in agreement with the reports of STRASBURGER (1873), BERNARD (1904) and VAN OOSTSTROOM (1948). In addition, it is in accordance with these authors that, if sporocarps are available, the species can also be identified by the presence of septate glochidia and the surface structure of the megaspore wall. The SEM micrographs of macrospores of *A. filiculoides (fig. 3a, b)* are very similar to those of specimens collected from Pleistocene soil layers (BERTELSEN 1972). The differences between the macrospores of the two species, as found in the present investigation, can be correlated with the drawings in the monograph by STRASBURGER (1873, Table VI, Fig. 97b and 101a), showing cross-sections of the spore walls. Strasburger observed that the protuberances in *A. filiculoides* seem to be surrounded by a firm "membrane" and that they are connected by more or less developed "bridge-like structures". In addition he noted that long hairs originate from the surface of the protuberances. The SEM micrographs show that the "membranes" and "bridge-like structures" are in fact dense clusters of hairs. In *A. caroliniana* this author illustrated an outer membrane of the megaspore wall with bud-like protuberances from which hairs originate. These bud-like protuberances probably represent the papillae as seen in the present study as well as the hair mass through which the papillae protrude.

The curly general appearance and the relatively large upper lobes of the leaflets of *A. filiculoides* with broad, membranous margins, are typical of luxuriantly growing plants. The length of the upper lobes was observed to be within the values as reported by VAN OOSTSTROOM (1948). In more diminutive modifications ("intermediate" forms) the length of the upper lobes of the leaflets is about the same as in the smaller species, and the hyaline margin is less pronounced.

Consequently these characteristics are unreliable for the taxonomic identification of "atypical" modifications in the field. In this respect the statement of CLAPHAM et al. (1962, p. 42) regarding the occurrence of *Azolla* in the British Isles is of interest ("*Azolla caroliniana* has been recorded as naturalized on insufficient evidence... but can only be certainly distinguished microscopically"). VAN OOSTSTROOM (1948) and LAWALREE (1964) described the shape of the upper lobes of the leaflets in *A. filiculoides* as ovate or broadly ovate, obtuse, and in *A. caroliniana* as ovate, narrowing towards the apex, obtuse or subacute, asymmetrical. These characteristics were not always consistent in the samples studied in the present investigation. A similar conclusion holds for the number of massulae per microsporangium, which Van Ooststroom

Table 4. Chemical composition of the water at sites of *Azolla* populations in The Netherlands. (COD = Chemical Oxygen Demand; TOC = Total Organic Carbon).

	A. filiculoides (typical f.)					Intermediate form					*A. cf. caroliniana*				
	Min.	Max.	Mean	N	Ref.	Min.	Max.	Mean	N	Ref.	Min.	Max.	Mean	N	Ref.
cond.	35 × 10	136 × 10	82 × 10	15	a	35.10	51.10	45.10	6	e	237	93 × 10	39 × 10	14	a
(K 25)	52 × 10	83 × 10	65 × 10	3	b						33 × 10	101 × 10	63 × 10	7	e
			46 × 10	40	c										
	32 × 10	250 × 10	90 × 10	5	d										
	40 × 10	397 × 10	200 × 10	25	e										
pH	6.9	7.8		15	a			8.2	1	e	6.7	7.3		14	a
	7.4	8.9		3	b						7.0	8.4		5	e
	7.1	7.9		5	d										
	7.1	8.6		25	e										
alkal.															
(meq/l)	3.4	8.2	6.5	3	a			2.8	1	e	2.4	4.0	2.9	5	a
	3.5	5.9	4.3	3	b						1.7	5.6	3.3	5	e
	3.4	8.3	5.7	5	d										
	1.2	7.2	4.6	25	e										
Cl^-	63	243	137	8	a	27	78	51	6	e	34	70	48	12	a
(mg/l)	76	126	100	3	b						38	220	85	7	e
	55	950	293		d										
	79	1040	449	25	e										
SO_4^{2-}	47	87	67	2	a			17	1	e	11	47	19	5	a
(mg/l)	25	350	138	25	e						5	62	38	5	e
$P.PO_4^{3-}$	0.17	0.47	0.33	3	a	0.11	1.27	0.48	5	e	0.07	1.8	0.7	5	a
(mg/l)	0.02	0.14	0.07	3	b						0.014	0.83	0.25	6	e
			0.14	40	c										
	0.015	1.9	0.24	24	e										
P-tot.	0.043	2.6	0.69	24	e			0.33	1	e	0.050	0.89	0.33	5	e
(mg/l)															

$N.NO_3^-$	0	tr.		3	a	0.1	1	e	0	tr.		5	a
(mg/l)	0	0.8		3	b				tr.	1.1	0.4	5	e
	tr.	0.9	0.13	24	e								
$N.NO_2^-$	0	tr.		3	a	0.05	1	e	0	tr.		5	a
(mg/l)	0	0.09		3	b				tr.	0.067	0.024	5	e
	0.005	0.15	0.050	24	e								
$N.NH_4^+$	0.3	4.7	3.0	3	a	0.01	1	e	0.4	10	2.9	5	a
(mg/l)	0.9	3.9	2.0	3	b				0.00	0.37	0.13	5	e
	0.00	7.0	0.92	24	e								
Na^+	21	66	44	2	d	31	1	e	29	119	74	5	e
(mg/l)	62	576	243	25	e								
K^+	0.5	7	4	2	d	26	1	e	tr.	49	15	5	e
(mg/l)	5	134	34	25	e								
Ca^{2+}	54	125	88	3	a	21	1	e	43	55	50	5	a
(mg/l)	74	106	92	3	b				21	56	39	5	e
	51	188	97	5	d								
	13	172	80	25	e								
Mg^{2+}	8	34	21	3	b	3	1	e	4	17	10	5	e
(mg/l)	3	41	16	5	d								
	4	71	37	25	e								
COD	32	124	81	8	e	28	1	e	59	60	60	2	e
(mg 0/l)													
TOC	13	65	34	17	e				16	28	20	3	e
(mg/l)													

Chemical composition of the water at Oostzaan (A. filiculoides small f.): pH 7.2; conductivity (K 25) 159 × 10; Cl^- 300, $P.PO_4^{3-}$ 0.019, P-tot. 0.076, $N.NO_3^-$ trace, $N.NO_2^-$ 0.023, $N.NH_4^+$ 0.47, SO_4^{2-} 150, Ca^{2+} 64, Mg^{2+} 29, Na^9 175, K 14 mg/l; alkalinity 2.4 meq/l; COD 17 mg/l.

legend of references

a = Segal (1966); b = De Lange (1967); c = De Lange (1972); d = Grabandt (1967); e = present investigation.

reported to be about 6 in *A. caroliniana* and 4 to 8 in *A. filiculoides*.

The differences in the green colour were found to be consistent, but are hardly useful for field identification. The differences in the red colour during the autumn are more conspicuous – especially when vegetations are viewed from a distance. On the other hand, the chromatographic analyses did not reveal any qualitative differences in anthocyanidin composition. Both species turned out to contain the rather rare anthocyanidins luteolinidin and apigenidin. The presence of the first compound in *A. filiculoides* had already been recorded by MEEUSE & DE VLAMING (1970, unpublished data). Differences in the relative quantities of the anthocyanidins, in the glycoside components, and in plant structure might be responsible for the observed colour differences.

As regards the factors inducing anthocyanin synthesis one of them is known to be radiation: plants growing at shaded sites remain green (see, *e.g.*, BENEDICT 1923; SVENSON 1944; DUBOIS 1967). Glass covering prevents the appearance of the red colouration in outdoor cultures (DUBOIS 1967). The present investigation indicated that the temperature and the chemical composition of the water are important as well.

The results of the translocation experiments are not conclusive. As *A. filiculoides* grew rapidly in one of the habitats of *A. cf. caroliniana* in North-West Overijssel, it appears that the composition of the water can hardly form a barrier for the expansion of this species. It may be presupposed that in this area, where the waters are less eutrophic and less brackish as compared to those of the western part of the country, the competitive ability of *A. cf. caroliniana* is greater. In the greenhouse it was observed that *A. filiculoides* became the dominant species in a mixed population on wet soil, especially towards the end of the summer when the days became shorter. This may imply that the disappearance of *A. cf. caroliniana* from the western part of the country is the result of a "crowding out" effect, possibly indirectly caused by an increased water pollution as was suggested by SEGAL (1966).

Marked fluctuations in the abundance of *Azolla* in successive years, as observed at the site of sample 10, is in accordance with the findings of, *e.g.*, KLINKENBERG (1915) and VANHECKE (1976), both authors suggesting a temporary depletion in the water of certain components as a possible cause.

The results of the chemical analyses of the composition of the water at the sites of either species are in accordance with earlier reports, as shown in *table 4*. It is clear that the two species show a marked difference in milieu preference as far as the water composition is concerned. The "intermediate" forms appear to occur in waters of a poorer ionic composition, which suggests that this modification is a pauperized form of the species *A. filiculoides*. To a lesser extent this may also account for the plants from Oostzaan which were growing in water with a below average ionic composition commonly found for well-developed forms, but with a higher ionic composition than the water in which "intermediate" forms were found.

The classification of the two species in The Netherlands has never been compared with the more recent work of SVENSON (1944) in which a new delim-

itation of the New World taxa of *Azolla* was proposed. In this context it is interesting to survey the literature on the systematics of the genus in more detail. LAMARCK originally described the genus in 1785, which he based on specimens of *A. filiculoides* from the Magellan area. Later MEYEN (1838) divided the genus into two genera, viz., *Rhizosperma* and *Azolla*, which were treated as subgenera by METTENIUS (1847) and all subsequent authors. The main differences between these two subgenera are the absence or presence of glochidia (none in *Rhizosperma*) and the number of floats on the macrospore (9 in *Rhizosperma* and 3 in *Azolla*). According to METTENIUS (1867) and STRASBURGER (1873) the subgenus *Rhizosperma* consists of two, mainly tropical species, *viz.*, *A. nilotica* Decne and *A. pinnata* R. Br., neither of which occurs on the American continent.

The first classifications of the *Azolla* species from the American continent were based only on vegetative characteristics. After the description of *A. filiculoides* by LAMARCK (1783), it was WILLDENOW who in 1810 described a second American species from the south-eastern United States, *viz.*, *A. caroliniana*. He distinguished the latter from *A. filiculoides* by the character: "*foliis imbricatis adpressis*" (with imbricate leaves adpressed to the stem) as against "*foliis patentibus*" (with spreading leaves) in *A. caroliniana*. Subsequently, more American species were described, such as *A. mexicana* by PRESL in 1845 from material which was collected by Schiede in Mexico in 1820 (the name *A. mexicana* had originally been given by SCHLECHTENDAL & CHAMISSO in 1830 without a description). After having studied *A. mexicana* material from the same Schiede collection METTENIUS (1847) gave a detailed characterization of the sporocarps, and identified the material as belonging to *A. caroliniana*, which he considered to be conspecific, so that *A. mexicana* became a synonym of *A. caroliniana*. The glochidia of the Schiede specimens were found to be septate, and the surface of the megaspore was relatively smooth. The illustrations by STRASBURGER (1873) of microsporocarps of *A. caroliniana* with septate glochidia are also based on herbarium material. The origin of these plants is not mentioned, and SVENSON (1944) believes that Strasburger had been influenced by the illustration of METTENIUS (1847).

The revision of the New World *Azolla* species by Svenson is primarily based on the septation of the glochidia, but morphological differences in vegetative parts and outer structure of the macrospore wall were also taken into account. In addition to *A. filiculoides*, of which he states that well-developed forms can be identified at a glance, he recognised another three species, *viz.*, *A. caroliniana*, *A. mexicana* and *A. microphylla*. The latter was originally described by KAULFUSS in 1824 from vegetative material collected in California. METTENIUS maintained it as a separate species in 1847, but in his last work on *Azolla*, which forms a part of "*Plantae tinneanae*" (1867), he considered it to be synonymous with *A. caroliniana*. According to SVENSON (1944), the glochidia of *A. filiculoides* and *A. caroliniana* are non-septate, in contrast to the septate glochidia in the two re-instated species. *A. mexicana* and *A. microphylla* are said to be distinguishable by the "pitted" megaspore wall of the former and

the "smooth" megaspore wall of the latter species, and to exhibit, in addition, differences in vegetative characters. Megasporocarps were absent from the material of *A. caroliniana* that Svenson had at his disposal.

As the glochidia of the species known in The Netherlands as "*A. caroliniana*" are septate, the identification is incorrect, judging by Svenson's criterion. The species could be either *A. mexicana* or *A. microphylla*. The distinction between these two species by Svenson is mainly based on the surface structure of the megaspore as seen under a microscope ("pitted" in *A. mexicana* and "smooth" in *A. microphylla*). In the present investigation this characterization proved to be very difficult to define and could not satisfactorily be correlated with the SEM micrographs of the Dutch material. On the other hand, the apparent absence of the equatorial girdle would point to *A. microphylla* as described in the earlier work of Mettenius, who reported that sporocarps of this species occasionally lack such a girdle. In this regard more detailed information on the anatomy of the macrospore apparatus in both species would be necessary to clarify the situation. An earlier suggestion of the occurrence of *A. mexicana* in The Netherlands next to *A. filiculoides* and *A. caroliniana* and intermediate in appearance between them was refuted by DUBOIS (1967). It is possible that the specimens studied by Dubois are comparable with the "intermediate" form of the present investigation.

Since Svenson's conclusions are based mainly on herbarium material, a comparative study of living plants from different regions of the American continent could help to clarify the complicated systematics of the *Azolla* species in question. GODFREY *et al.* (1961) found both septate and non-septate glochidia in the same massula of a strain from Florida (from where only *A. caroliniana* has been recorded), and consequently queried the classification of Svenson. Differences in the pattern of septation of the glochidia between an *A. caroliniana* strain from North America and Dutch material had previously been noticed by BERNARD (1904). Moreover, the consistent differences in general morphology between the Dutch samples and the one of "*A. caroliniana*" from Venezuela might be relevant.

In the light of these considerations the name *A. caroliniana* for the smaller species occurring in The Netherlands is questionable until more precise information on the American genotypes has become available.

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