EVALUATION OF SALT TOLERANCE IN *AZOLLA*

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ABSTRACT

The aquatic fern Azolla and the symbiotically associated cyanobacteria (blue-green alga) Anabaena azollae is a highly productive system for nitrogen fixation. The tolerance of several species and ecotypes of Azolla to increasing concentrations of salinity as NaCl was compared in environmental chamber studies using nitrogen-free medium. Growth of Azolla mexicana collected near Corrales, New Mexico, was not significantly inhibited in medium containing 50 milliequivalent (meq)/liter NaCl compared to the control without added NaCl. At 100 meq/liter NaCl yield of Azolla decreased to 42 percent of the control. Little growth occurred at 150 meq/liter NaCl. NaCl concentrations of 50 and 100 meq/liter correspond to very high salinity hazard irrigation waters that are unsuitable for ordinary irrigation use. Based on this investigation some saline waters appear to have application for the hydroponic production of Azolla as a nitrogen-rich organic material with potential for use as a feed or compost material.

Other species of Azolla and also stocks of A. mexicana collected from slightly saline waters in New Mexico were tested for NaCl tolerance. Several of these stocks were less salt tolerant than the Corrales A. mexicana and none demonstrated greater salt tolerance. Azolla was collected from the Bernardo, New Mexico area in water with a conductivity of 1.5 mmhos/cm; however, Azolla was not observed in more saline waters in either the Rio Grande or Pecos River valleys.

Azolla mexicana was found to be more tolerant of NaCl salinity than salinity imposed by addition of other salts including $MgSO_4$, $MgCl_2$, K_2SO_4, KCl, Na_2SO_4, or $CaCl_2$.

When *A. mexicana* was provided with fixed nitrogen in the medium, it was able to grow in 100 meq/liter NaCl without growth inhibition and at 150 meq/liter with 68 percent of the control yield. The symbiotic *Anabaena* and nitrogen fixation thus appear to be more sensitive to NaCl inhibition than the host fern when supplied with fixed nitrogen.

Key words: *Azolla*, salt tolerance, saline water, nitrogen fixation, hydroponics

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INTRODUCTION AND JUSTIFICATION

The small floating fern *Azolla* and the symbiotically associated cyanobacteria (blue-green alga) *Anabaena azollae* fix large amounts of atmospheric nitrogen gas, equaling or surpassing nitrogen fixation by highly productive legumes such as alfalfa. The nitrogen fixation potential of *Azolla* has been estimated to range from 313 (Evans and Barber 1977) to over 600 kg of nitrogen/ha/yr under optimum tropical conditions, suggesting that this symbiosis is probably the most productive biological nitrogen fixing system known. *Azolla* has been associated with rice paddies in China for centuries and has been of major importance in maintaining adequate nitrogen for rice production (Singh 1981). In Vietnam, *Azolla* has been managed so it provides nitrogen for high rice yields. Elsewhere in Asia, as well as in the United States, *Azolla* is being evaluated as a component of rice culture systems (Talley and Rains 1980a, 1980b). In addition to the importance of *Azolla* in the nitrogen economy of natural ecosystems and rice paddies, *Azolla* has been used as a general compost material and as a feed for fish, poultry, and livestock (Moore 1969).

The genus *Azolla* consists of six species of small aquatic ferns that have a worldwide distribution in tropical and temperate environments (Singh 1981). *Azolla mexicana*, *A. filiculoides*, and *A. caroliniana* occur in North America (Moore 1969); however, only *A. mexicana* has been reported in New Mexico (Martin and Hutchins 1980). All species of *Azolla* are associated with a symbiotic cyanobacterium, *Anabaena azollae*, which is enclosed in internal cavities on the underside of the dorsal leaves (Moore 1969). The dorsal leaves are aerial and contain chlorophyll, occurring just above the surface of the water (Peters 1977, 1978). Nitrogen is fixed in the numerous heterocysts in the filaments of Anabaena and released in the cavity for utilization by the host. The symbiotic cyanobacteria can, via fixation, supply Azolla with its total nitrogen requirement. If the symbiotic cyanobacteria is artificially separated from the host, Azolla is demonstrated to be totally dependent on fixed nitrogen in the culture medium (Peters and Mayne 1974). Unlike legumes and free living cyanobacteria, the Azolla symbiosis continues to fix nitrogen even when combined nitrogen is present in the culture medium (Peters 1977).

Considering the abundance of saline water in New Mexico and elsewhere in the southwestern United States, a salt tolerant variety of Azolla would be of considerable significance as a potential crop for growth in these waters. Possible uses of salt tolerant Azolla in the southwestern United States might be as a general compost material and as a feed for aquaculture systems, poultry, and livestock. Stewart (1980) indicates that a salt tolerant type of A. pinnata var. imbricata occurs in Vietnam suggesting that variation in salt tolerance may also occur in temperate region Azolla.

The overall objectives of this research were to evaluate the salt tolerance of Azolla and to make selections for Azolla with enhanced salt tolerance. The tolerance of several species and ecotypes of Azolla to increasing concentrations of salinity as NaCl was compared in growth chamber studies. The effect of various salts on growth of Azolla was evaluated in experiments with A. mexicana collected near Corrales, New Mexico. Two approaches to the selection of Azolla with enhanced salt tolerance were utilized:

1. A search was conducted for Azolla in habitats with saline surface waters for evaluation of salt tolerance in growth chamber studies.

2. Large numbers of *Azolla* plants were cultured in salinized hydroponic medium which was lethal to most of the plants. Growth of surviving plants was then evaluated in hydroponic medium at various salinities.

These salt tolerance experiments were conducted in hydroponic medium without fixed nitrogen; hence growth was dependent on nitrogen fixation. Several experiments were also conducted using medium containing fixed nitrogen in order to evaluate the effect of salinity on growth when nitrogen fixation was not obligatory.

MATERIALS AND METHODS

Azolla Stocks

Azolla mexicana used in many experiments in this investigation was collected in May 1984 from the Corrales Drain just west of the Rio Grande bridge on New Mexico Highway 46 in Bernalillo County, New Mexico. *Azolla* was also collected at other locations in the Rio Grande Valley as noted in subsequent sections of this report. Field collections of *Azolla* were washed in tap water to remove invertebrates and particulate debris. The ferns were then surface sterilized with 5.0 percent Clorox for 20 min to eliminate remaining invertebrates and contaminating algae. Clorox was then washed off with tap water. *Azolla* surviving the surface sterilization treatment resumed growth after several days in the growth medium.

The following *Azolla* stocks were obtained from the collection of Drs. D. W. Rains and Madeline Ames of the Department of Agronomy and Range Science, University of California, Davis, California.

Species	Collection Site	U.C. Davis Identification #
A. caroliniana	Botany Dept. greenhouse, Madison, Wisconsin	11
A. filiculoides	Walker Lake, Mineral Co., Nevada	15
A. filiculoides	Owens River, Mono Co., California	17
A. pinnata	Infugo, Philippines (IRRI No. 4)	52
A. caroliniana	Interdunal area at Naggshead Wood, Outer Banks, North Carolina	100

Plant Culture and Growth Experiments

Azolla was grown in a nitrogen-free medium similar to that used by Johnson et al. (1966). The nutrient solution consisted of the macronutrients in mmoles/liter: $MgSO_4$, 0.50; KH_2PO_4, 0.20; K_2HPO_4, 0.05; K_2SO_4, 0.49; $CaCl_2$, 0.13; $CaSO_4$, 1.00; and the micronutrients in µmoles/liter: B, 5.77 as H_3BO_3; Mn, 1.13 as $MnSO_4$; Zn, 0.19 as $ZnSO_4$; Cu, 0.08 as $CuSO_4$; Mo, 0.05 as $NaMoO_4$; Co, 0.017 as $CoCl_2$; and Fe, 25 as FeEDTA. When *Azolla* was grown with fixed nitrogen, this medium was modified by the addition of 2 mmoles/liter $Ca(NO_3)_2$ and $CaSO_4$ was omitted. The medium containing fixed nitrogen was autoclaved prior to use to assure that contaminating algae were eliminated.

Cultures of *Azolla* were maintained in the greenhouse and also under continual fluorescent lights in the laboratory; however, cultures were allowed to adapt to the light and temperature conditions of the growth chamber prior to use in experiments. Growth experiments were conducted in a plant growth chamber (Sherer CEL - 3714) with a 16 h light cycle at 25°C and an 8 h dark cycle at 22°C. Light was provided by cool white fluorescent lamps and incandescent bulbs at an intensity ranging from 195 to 370 $\mu Es^{-1}m^{-2}$ at plant height.

Azolla growth experiments without fixed nitrogen were conducted in 400-ml beakers containing 200 ml of medium. Growth experiments with fixed nitrogen supplied were conducted in 125-ml Erlenmeyer flasks containing 60 ml of medium. Beakers were covered with plastic petri dish covers and flasks with glass beakers to limit evaporation and to exclude dust.

Experiments were initiated by addition of 23-27 mg (fresh weight) of *Azolla* plants to beakers or flasks of medium. Redistilled water was added to vessels as necessary to maintain the initial volume. Cultures were usually harvested from the nitrogen-free medium after 16 days and from the medium containing combined nitrogen after 28 days. Fresh weights were determined after first rinsing plants in distilled water and then thoroughly blotting the plants. In most experiments total plant weight was determined; however, in certain experiments frond (shoot) and root weights were determined separately. Dry weights were determined after samples were dried for 24 h at 60-70°C. Dry weight data were analyzed statistically using analysis of variance and means were separated by the Student-Newman-Keuls multiple range test at the 0.05 level of significance.

Analysis of Plant Tissue and Water Samples

Nitrogen (reduced nitrogen, excluding nitrate) was determined by titration of ammonia after distillation from digested samples using a semi-micro Kjeldahl procedure. Selected oven-dried samples were subjected to acid digestion and analyzed for cations by atomic absorption spectrophotometry. Selected samples were also extracted with water using a ground glass tissue homogenizer for chloride analysis using a Technicon Autoanalyzer. Free proline was determined on separated fronds and roots following homogenization of fresh tissue in an Omnimixer in cold methanol:chloroform:water (120:50:30). Particulate matter was removed by centrifugation and filtration and proline was determined spectrophotometrically by the acid ninhydrin method (Bates et al. 1973).

Nitrogen fixation by Azolla was measured by the acetylene reduction method (Burris 1974). The acetylene reduction assay was conducted using 200 mg samples of the fern in 25 ml serum vials containing 2 ml of the appropriate salinized growth medium in an atmosphere of 10 percent acetylene in air. The vials were incubated in the growth chamber for 30 min under the usual daytime light and temperature conditions and 0.5 ml gas samples were removed for measurement of ethylene. Ethylene was measured using a Dorhmann 2460 gas chromatograph with a flame ionization detector fitted with a 9 ft x 1/8 in diameter column containing Porapak R. Column temperature was maintained at 40°C and helium gas was used as the carrier. Similar assays were conducted without injection of acetylene as controls.

The number of *Anabaena* cells in the fronds was determined in one experiment. Fronds were separated from the roots and 30 mg (fresh weight) of fronds were homogenized in 0.3 ml of 0.01 *M* phosphate buffer at pH 6.5. A sample of the suspension was placed in a hemocytometer and the number of *Anabaena* cells was determined using a microscope.

The conductivity of field collected water samples and salinized nutrient solutions was measured using a conductivity bridge.

RESULTS AND DISCUSSION

Effects of NaCl Salinity on Growth of Azolla

The response of Azolla mexicana collected near Corrales, New Mexico to NaCl salinity is shown in table 1 and figure 1. The maximum yield as dry weight was obtained at 10 meq/liter NaCl with 125 percent of the dry weight of the control grown without added NaCl. At 50 and 100 meq/liter the relative dry weight decreased to 88 percent and 47 percent of the control yield respectively. At a NaCl concentration of 150 meq/liter, relative yield decreased to 14 percent of the control. Further decreases in growth occurred at NaCl concentrations of 200 meq/liter or greater. These results were confirmed in a second experiment with A. mexicana from Corrales, New Mexico (table 1, experiment 2). Again growth stimulation occurred at 10 meq/liter NaCl, growth was slightly depressed at 50 meq/liter, and less than 50 percent of the control yield as dry weight was obtained at 100 meq/liter.

Plants cultured at 0 and 10 meq/liter NaCl were uniformly green in color. As the NaCl concentration increased to 50 meq/liter a few fronds exhibited a red or brown color. At 100 meq/liter growth was substantially inhibited and 10-20 percent of the fronds were red or brownish-green in color. At 150 meq/liter little growth occurred and most fronds were red in color. Only a small amount of growth occurred at 200 meq/liter (table 1) and the fronds were brown in color.

The nitrogen content of Azolla tissue (table 1, experiment 1) was 5.7 percent on a dry weight basis without added NaCl and decreased slightly to 5.4 percent at 10 meq/liter and 4.8 percent at 50 meq/liter NaCl. At 100 meq/liter the nitrogen content decreased to 3.6 percent and at 150 meq/liter

Table 1.

The effect of NaCl on growth and nitrogen content of *Azolla mexicana*

	NaCl meq/liter	Fresh Wt mg	Dry Wt mg	Relative Yield (%)	Dry Wt %	Nitrogen %
EXP 1 (N=3)	0	1487±164.9*	83.8±9.21ab**	100	5.64	5.70±0.12
	10	1956±513.6	105.2±27.53a	125.5	5.38	5.38±0.26
	50	1303±269.6	73.7±14.68b	88.0	5.66	4.78±0.13
	100	492±47.8	39.7±5.25c	47.4	8.07	3.56±0.11
	150	57.7±13.32	11.5±2.72d	13.7	19.93	1.03±0.14
	200	42.0±5.20	10.2±0.92d	12.2	24.38	1.36±0.16
	250	19.0±1.73	5.6±0.77d	6.7	29.37	1.90±0.19
EXP 2 (N=4)	0	792±118	54.2±5.4b	100	6.84	5.14±0.09
	10	1155±296	73.8±17.9a	136.2	6.39	4.68±0.39
	50	703±169	45.8±9.7b	84.5	6.51	4.27±0.55
	100	200±106	19.5±7.0c	36.0	9.75	3.33±0.25
	150	49.5±6.8	9.0±0.8c	16.6	18.18	2.10±0.38
	200	20.8±6.1	5.0±1.2c	9.2	24.04	2.47±0.34

* Standard deviation

** Means for each experiment followed by a common letter do not differ significantly at the 0.05 level.

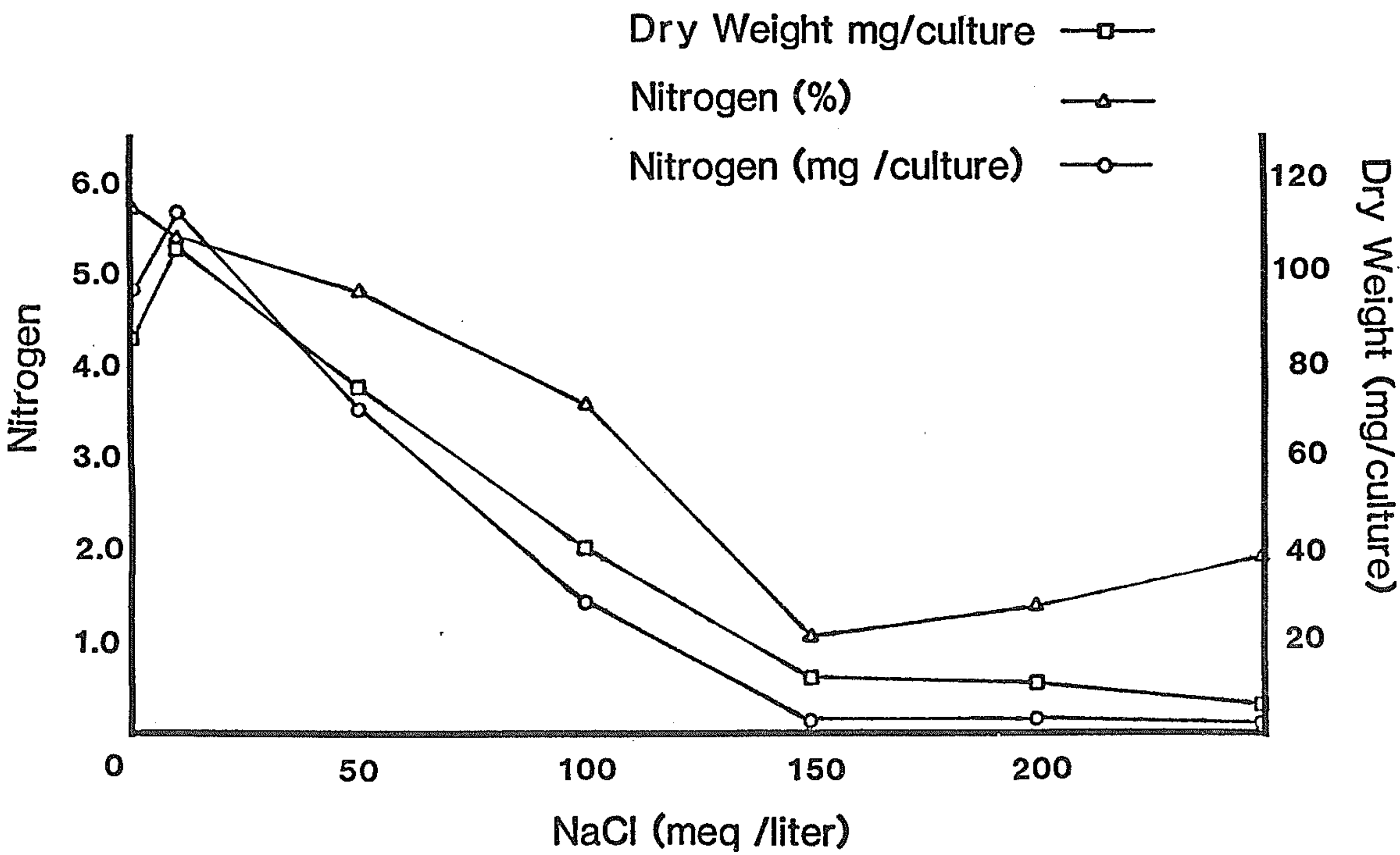

Figure 1. Effect of NaCl salinity on growth and nitrogen content of *A. mexicana*

or higher concentrations of NaCl the percent nitrogen content was greatly reduced as was yield. These effects were confirmed in the second experiment tabulated in table 1.

Azolla obtained from the University of California at Davis and Azolla collected at two other locations in the Rio Grande Valley of New Mexico were evaluated for growth at various concentrations of NaCl (table 2). Relative yields on a dry weight basis for cultures obtained from the University of California and A. mexicana from Corrales, New Mexico, are depicted in figure 2. Azolla caroliniana from Madison, Wisconsin and A. mexicana from Corrales, New Mexico, appear to be most tolerant based on growth in the range of 10-50 meq/liter NaCl while A. pinnata, A. caroliniana from a coastal dune area in North Carolina, and A. filiculoides from Mono County, California, were least tolerant. Growth of A. filiculoides from Walker Lake, Nevada, was intermediate.

It was hypothesized that A. filiculoides from Walker Lake, Nevada, and A. caroliniana from North Carolina might have enhanced salt tolerance because they were collected from waters having significant salinity (Rains, D.W. and M. Ames, personal communication); however, they appeared less salt tolerant than A. filiculoides from Madison, Wisconsin, or A. mexicana from Corrales, New Mexico. It is possible that genetically based salt tolerance may have been lost from these ecotypes during prolonged greenhouse cultivation on non-saline medium.

Attempts were made to collect Azolla from saline waters in the Pecos River Valley between Carlsbad and Roswell, New Mexico. Azolla was not observed in either the saline or non-saline waters visited in the Pecos River Valley although A. mexicana is reported to occur in the Pecos Valley

Table 2.

The effect of NaCl on growth and nitrogen content of several species of *Azolla* (N=4)

Species	NaCl meq/liter	Fresh Wt mg	Dry Wt mg	Relative Yield (%)	Dry Wt %	Nitrogen %
A. filiculoides (15)*	0	1173±263	73.0±15.7ab+	100	6.22	5.24±0.30
	10	1403±318	82.8±16.2a	113.4	5.90	4.74±0.08
	50	868±337	55.8±18.9b	76.4	6.43	3.70±0.44
	100	307±165	26.5±10.7c	36.3	8.63	3.14±0.29
	150	82.8±21.7	12.5±2.4c	17.1	15.10	2.12±0.26
	200	29.8±6.9	6.2±1.3c	8.5	20.81	2.20±0.24
A. filiculoides (17)	0	1003±114	66.0±5.9a	100	6.58	5.37±0.21
	10	1014±215	59.5±10.6a	90.2	5.87	4.68±0.19
	50	574±248	40.8±16.9b	61.8	7.11	3.32±0.54
	100	188±50	21.5±4.1c	32.6	11.44	2.18±0.33
	150	68.5±21.5	12.2±3.1c	18.5	17.81	1.87±0.17
	200	29.8±1.7	6.8±0.5c	10.3	22.82	1.71±0.18
A. caroliniana (11)	0	894±248	52.8±11.6b	100	5.91	4.64±2.01
	10	1151±182	69.2±9.6a	131.1	6.01	4.45±0.16
	50	603±80	46.5±5.5b	88.1	7.71	3.84±0.09
	100	178±25	21.5±2.4c	40.7	12.08	2.74±0.15
	150	55.0±16.6	12.5±2.4cd	23.7	22.73	1.74±0.33
	200	29.0±9.9	6.8±2.1d	12.9	23.45	1.80±0.31
A. caroliniana (100)	0	956±254	53.5±11.6a	100	5.60	3.89±0.45
	10	860±143	49.0±6.1a	91.6	5.70	3.75±0.67
	50	331±122	29.2±7.4b	54.6	8.82	2.89±0.19

Table 2. (continued)

A. caroliniana (100) (continued)	100	137±44	20.2±4.1bc	37.8	14.74	1.95±0.14
	150	44.5±7.8	11.0±0.8cd	20.6	24.72	1.48±0.07
	200	28.8±7.2	8.0±1.4d	15.0	27.78	1.34±0.08
A. pinnata (52)	0	1046±331	62.0±17.9a	100	5.93	4.42±0.91
	10	1210±320	68.0±16.8a	109.7	5.62	5.04±0.31
	50	540±159	38.2±9.0b	61.6	7.07	4.05±0.15
	100	173±82	19.0±7.4c	30.6	10.98	2.61±0.25
	150	45.0±4.5	8.8±1.3c	14.2	19.56	1.90±0.12
	200	24.8±2.8	4.5±0.6c	7.3	18.15	2.26±0.19
Azolla sp. (Bosque del Apache, NM)	0	826±196	52.5±11.3a	100	6.36	5.06±0.37
	10	817±188	48.2±10.2a	91.8	5.90	4.26±0.51
	50	444±55	35.2±3.3b	67.0	7.93	3.29±0.84
	100	169±81	21.2±5.5c	40.4	12.54	2.28±0.38
	150	42.0±12.5	8.5±2.4d	16.2	20.24	2.06±0.07
	200	24.2±3.4	6.0±0.8d	11.4	24.79	2.55±0.37
Azolla sp. (Bernardo, NM)	0	1441±237	85.8±13.2a	100	5.95	5.58±0.43
	10	1276±172	70.2±10.0b	81.8	5.50	4.95±0.44
	50**	890±85	75.3±3.5ab	87.8	8.46	2.95±0.19
	100	114±65	14.5±5.4c	16.9	12.72	2.30±0.43
	150	52.2±11.2	10.0±1.4c	11.6	19.16	1.87±0.13
	200	34.2±7.9	8.5±1.9c	9.9	24.85	1.98±0.34

* Identification number from the collection of Drs. D.W. Rains and M. Ames, Department of Agronomy and Range Science, University of California, Davis

** N=3

+ Means for each species or population followed by a common letter do not differ significantly at the 0.05 level.

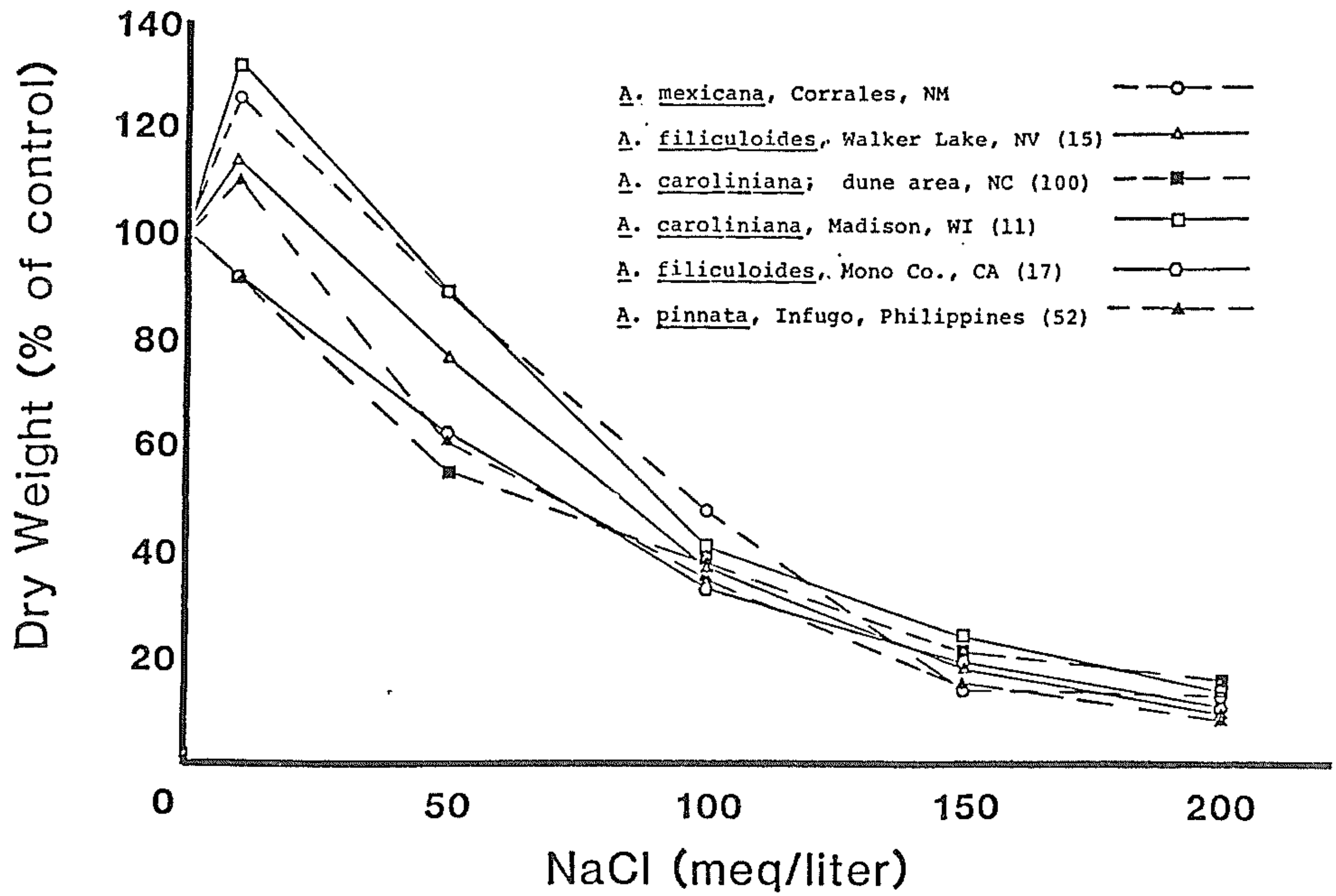

Figure 2. Effect of NaCl salinity on growth of several species of *Azolla*

(Martin and Hutchins 1980). *Azolla* was collected at Bosque del Apache and Bernardo in the Rio Grande Valley, New Mexico. The conductivity of water from the Bosque del Apache and Bernardo sites was 1.24 and 1.48 mmhos/cm respectively. Water with a conductivity of 0.25-0.75 mmhos/cm has a medium salinity hazard while water with a conductivity of 0.75-2.25 mmhos/cm has a high salinity hazard (USDA 1954).

Azolla from Bosque del Apache and Bernardo were not stimulated by addition of 10 meq/liter NaCl; however, relative dry weights at 50 meq/liter NaCl suggested that their salt tolerance was similar to that of *A. mexicana* collected from water having a conductivity of 0.33 mmhos/cm at Corrales, New Mexico. The salt tolerance of *Azolla* from Bosque del Apache and from Bernardo was evaluated six months and one month respectively after collection from the field. It is unlikely that any genetically based salt tolerance would be lost in one or even six months of growth on non-saline medium in the laboratory. Therefore, these collections from high salinity hazard waters appear to be no more salt-tolerant than *Azolla* from medium salinity hazard water at Corrales, New Mexico.

While the significance of salt tolerance to *Azolla* production is widely recognized (e.g. Silver and Schröder 1984) only a few studies have evaluated the ability of *Azolla* to grow in saline media. Haller et al. (1974) reported that the yield of *A. caroliniana* decreased to 59 percent of the control dry weight when salt water was added to the culture medium to obtain a concentration of about 57 meq/liter NaCl. In a study of *A. mexicana*, Holst and Yopp (1979) found that the growth rate of *Azolla* decreased at 68 meq/liter NaCl to 76 percent of the low NaCl control growth rate based on fresh weight measurements. Thus the result of the present investigation are in general agreement with the limited reports in the literature concerning the ability of Azolla to grow in NaCl containing media.

Effects of Other Salts on Growth of Azolla

The effects of salts other than NaCl on growth of *A. mexicana* were investigated in two series of experiments. Table 3 presents results of growth experiments when Azolla was cultured with Cl or SO_4 salts of Na, K, Mg and Ca ranging up to 200 meq/liter. The yield data, expressed as percentages of the dry weight of the control without added salt in each experiment, are depicted for Cl and SO_4 salts in figures 3 and 4 respectively. The results for growth with NaCl salinity from table 1 (experiment 1) are included in both figures for comparison. To distinguish specific salt effects from general osmotic effects, the salt concentrations in figures 3 and 4 are expressed as milliosmoles/liter.

Except at the lowest salt concentrations where effects on yield were either slightly stimulatory (NaCl and $CaCl_2$) or slightly inhibitory ($MgCl_2$ and KCl), the salts KCl, $CaCl_2$, and $MgCl_2$ were much more damaging than NaCl (figure 3).

When SO_4 salts are compared to NaCl, it is apparent that, except for very low concentrations, K_2SO_4 and $MgSO_4$ were much more inhibitory than NaCl (figure 4). Concentrations of Na_2SO_4 up to 75 milliosmoles/liter slightly inhibited Azolla growth; however, a concentration of 150 milliosmoles/liter appeared to be much more inhibitory than a similar concentration of NaCl. Because of the low solubility of $CaSO_4$, the maximum concentration of this salt that could be prepared was 28 milliosmoles/liter, which did not significantly decrease Azolla yield.

Table 3.

The effect of various salts on growth and nitrogen content of <u>Azolla mexicana</u> (N=4)

Salt	meq/liter	Fresh Wt mg	Dry Wt mg	Relative Yield (%)	Dry Wt %	Nitrogen %
KCl	0	1085±160.5	61.2±7.5a+	100	5.64	4.65±0.39
	10	855±113.9	47.5±5.3b	77.6	5.56	4.72±0.29
	50	318±156.8	25.2±9.7c	41.2	7.92	1.96±0.80
	100	10.2±2.87	3.2±0.5d	5.2	31.37	3.40±0.46
	150	4.5±3.11	0.8±0.3d	1.3	17.78	5.67*
	200	6.2±0.96	1.0±0d	1.6	16.13	5.00*
K_2SO_4	0	1070±335.3	64.5±17.7a	100	6.03	4.55±0.49
	10	735±220.5	43.8±12.7b	67.9	5.96	3.97±0.26
	50	254±25.3	21.0±3.7c	32.6	8.27	1.77±0.29
	100	17.5±7.55	2.8±0.5d	4.3	16.00	3.62±0.80
	150	12.5±3.32	1.8±0.5d	2.8	14.40	3.71*
	200	9.5±3.32	1.0±0d	1.6	10.53	5.25*
Na_2SO_4	0	1292±352.6	75.0±19.20a	100	5.80	5.15±0.32
	10	1015±113.4	57.8±9.07a	77.1	5.69	5.34±0.79
	50	965±372.2	56.2±21.8a	74.9	5.82	4.76±0.39
	100	138±27.3	15.0±1.63b	20.0	10.87	1.97±0.18
	150	51.5±7.94	8.5±1.29b	11.3	16.50	1.52±0.26
	200	42.0±10.61	7.5±2.65b	10.0	17.86	1.89±0.38
$MgCl_2$	0	1006±283.6	57.8±16.19a	100	5.75	4.62±0.308
	10	971±119.8	51.8±6.50a	89.6	5.33	4.59±0.39
	50	298±34.5	23.0±2.00b	39.8	7.72	2.36±0.165

Table 3. (continued)

$MgCl_2$ (cont'd)	100	43.8±8.06	9.0±0.82c	15.6	20.55	1.61±0.274
	150	21.5±3.32	5.2±0.96c	9.1	24.19	2.50±0.556
	200	17.2±3.69	3.0±0.82c	5.2	17.44	3.47±0.452
$MgSO_4$	0	1017±338.3	58.8±17.95a	100	5.78	5.06±0.78
	10	872±94.3	51.5±8.35a	87.6	5.91	4.80±0.15
	50	212±56.5	18.5±4.43b	31.5	8.73	1.95±0.21
	100	59.8±4.35	10.8±0.50b	18.4	18.06	1.34±0.08
	150	41.0±1.63	7.8±0.50b	13.3	19.02	1.59±0.13
	200	27.8±3.20	5.0±0.82b	8.5	17.99	2.16±0.30
$CaCl_2$	0	1220±141.0	70.7±7.07b	100	5.80	5.06±0.408
	10	1468±321.7	84.9±16.32a	120.1	5.78	4.73±0.527
	50	212±39.5	21.6±3.13c	30.5	10.19	2.60±0.089
	100	34.5±12.15	8.3±2.34d	11.7	24.06	1.90±0.603
	150	17.8±2.75	5.6±0.83d	7.9	31.46	2.20±0.324
	200	19.0±4.76	3.8±1.13d	5.4	20.00	3.19±1.536
$CaSO_4$	0	1058±191.9	62.5±8.6	100	5.91	4.25±0.49
	10	1101±363.3	68.0±20.1	108.8	6.18	3.86±0.19**
	28	874±146.5	57.5±7.8	92.0	6.58	3.31±0.90

* Four replicate samples combined for analysis

** Mean of two samples

+ Means for each salt followed by a common letter do not differ significantly at the 0.05 level.

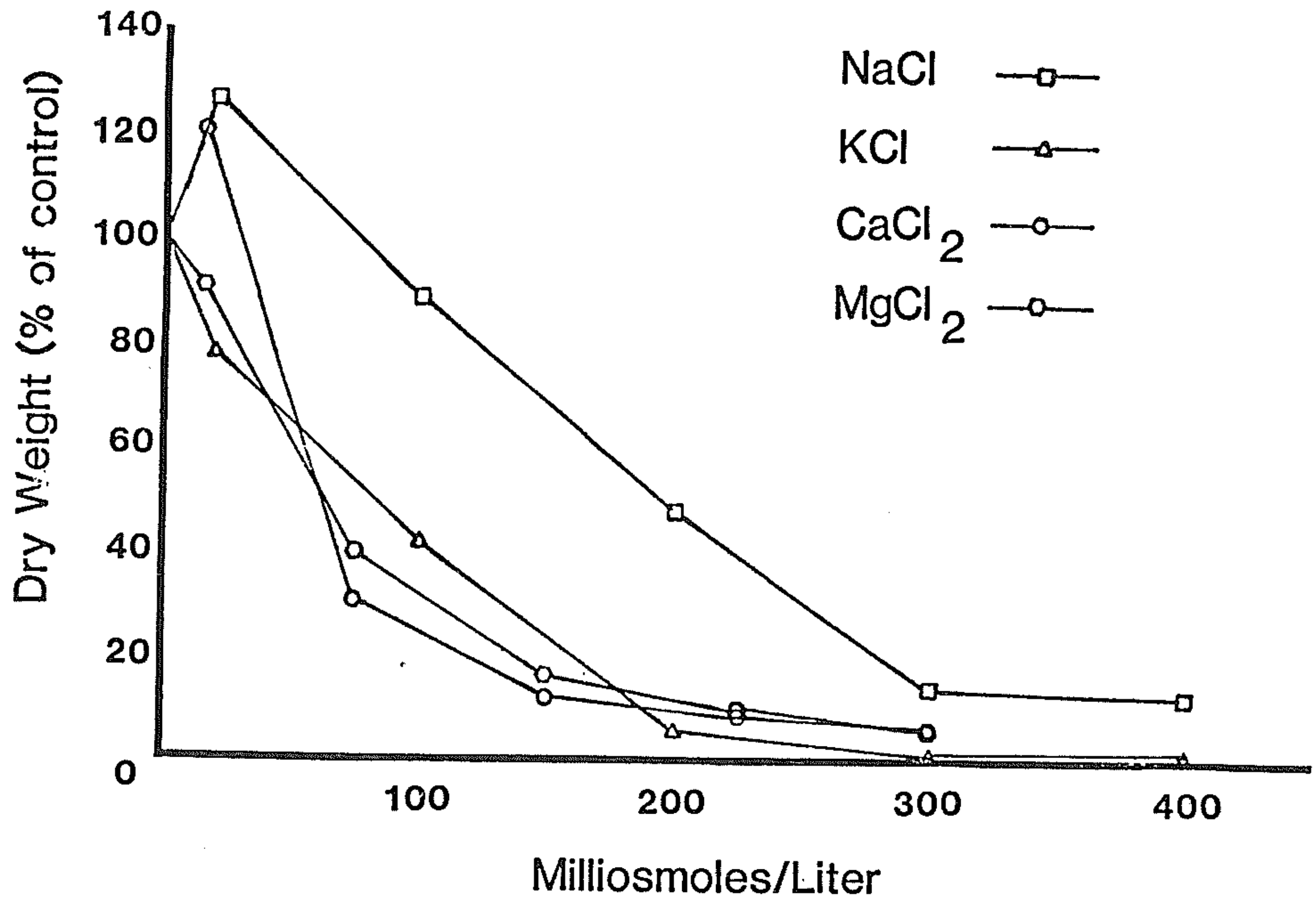

Figure 3. Effect of NaCl, KCl, $CaCl_2$, and $MgCl_2$ on growth of A. mexicana

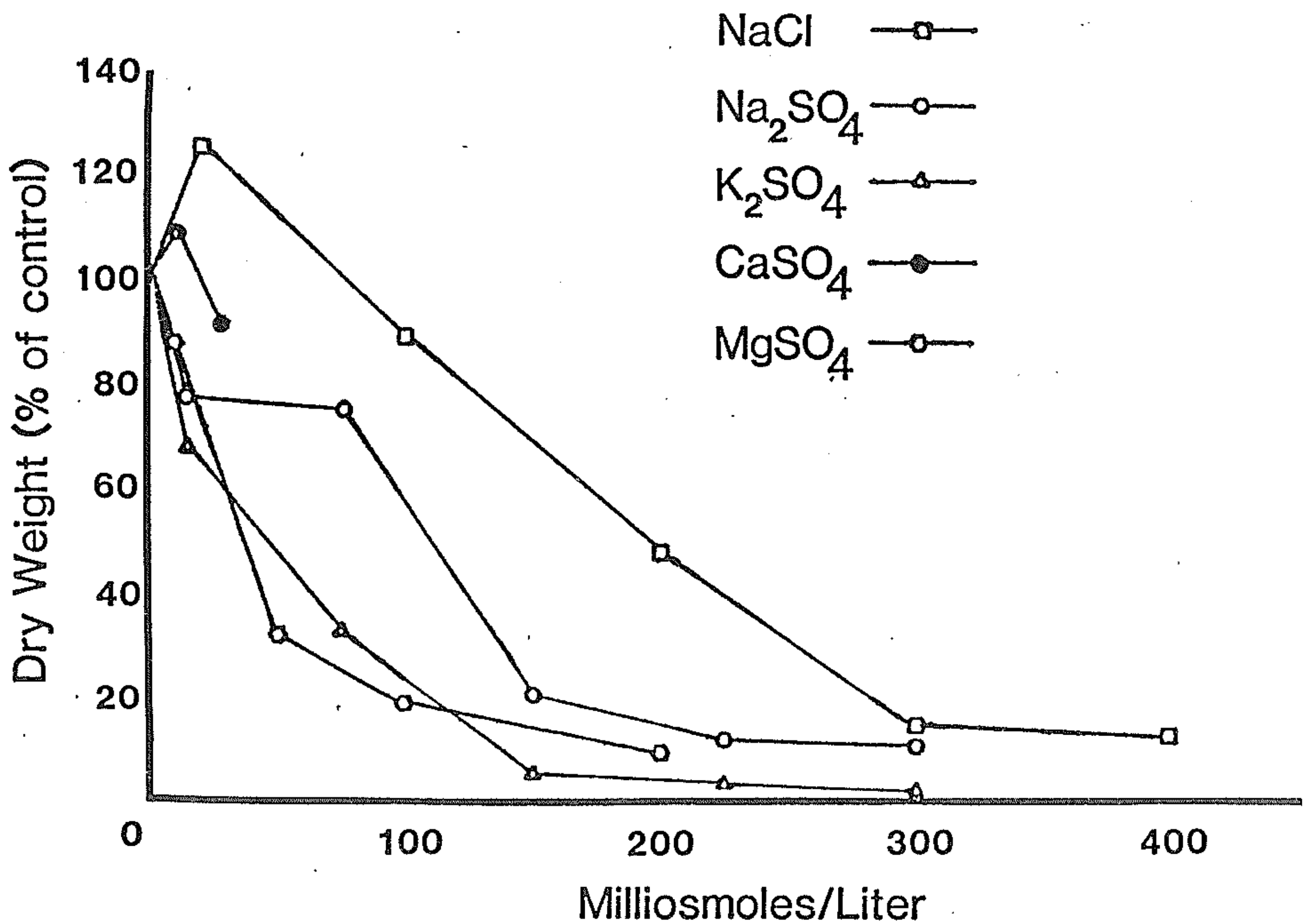

Figure 4. Effect of NaCl, Na_2SO_4, K_2SO_4, $CaSO_4$, and $MgSO_4$ on growth of *A. mexicana*

The percent nitrogen concentration of Azolla tissue was essentially unaffected by 10 meq/liter of any salts (tables 1 and 3). At a concentration of 50 meq/liter the percent nitrogen concentration decreased to a range of 1.77 percent to 2.60 percent except in the NaCl and Na_2SO_4 treatments where the percent nitrogen remained at about 4.7 percent (tables 1 and 3). At 100 meq/liter Na_2SO_4 the percent nitrogen content decreased to 1.97 percent while at 100 meq/liter NaCl the percent nitrogen decreased to 3.56 percent (table 1, experiment 1). The percent nitrogen content as well as the relative yield is a suitable indication of the toxicity of different salts.

Experiments were conducted using equal osmolar concentrations of various salts to more clearly demonstrate the differential effects of various salts on growth of Azolla (table 4 and figure 5). Because the maximum yield of A. mexicana was obtained with addition of 10 meq/liter NaCl, this low concentration of NaCl was present in the control medium and also in medium with addition of 50, 100 and 200 milliosmoles/liter of the indicated salts. The inhibitory effects of salts on relative dry weight of Azolla at various osmolarities may be summarized as follows:

50 milliosmoles/liter: Na_2SO_4, NaCl, $MgCl_2$ < KCl, K_2SO_4, $CaCl_2$ < $MgSO_4$

100 milliosmoles/liter: NaCl, Na_2SO_4, KCl < $CaCl_2$ < $MgCl_2$ $MgSO_4$ < K_2SO_4

200 milliosmoles/liter: NaCl < $CaCl_2$, Na_2SO_4, $MgSO_4$, $MgCl_2$ < KCl, K_2SO_4

As has been observed in studies of other non-halophytic plant species, Azolla appears to be most tolerant at higher concentrations of NaCl while $CaCl_2$ and Na_2SO_4 are more inhibitory and $MgSO_4$, $MgCl_2$, K_2SO_4, and KCl are most damaging. Clearly, specific salt effects are important, and Azolla growth is not primarily limited by non-specific osmotic effects.

Table 4.

The effect of equal osmotic concentrations of various salts on growth of Azolla mexicana (N=4)*

Concentration (mos)**		Fresh Wt mg	Dry Wt mg	Relative Yield (%)	Dry Wt %
50	control	788±195	54.9±12.8	100.0	6.97
	NaCl	512±102	39.6±7.9a+	72.1	7.73
	Na_2SO_4	564±179	42.8±11.2a	78.0	7.59
	KCl	394±27	28.8±3.7ab	52.5	7.31
	K_2SO_4	364±140	28.2±8.4ab	51.4	7.75
	$CaCl_2$	295±111	27.2±8.5ab	49.5	9.22
	$MgCl_2$	477±95	38.0±4.4a	69.2	7.97
	$MgSO_4$	188±49	18.8±6.0b	34.2	10.00
100	control	646±152	49.2±9.8	100.0	7.62
	NaCl	446±127	33.2±8.1a	67.5	7.44
	Na_2SO_4	323±54	27.2±3.6a	55.3	8.42
	KCl	336±227	27.0±15.7a	54.9	8.04
	K_2SO_4	33.2±8.8	5.0±0.8c	10.2	15.06
	$CaCl_2$	198±28	20.0±2.2ab	40.6	10.10
	$MgCl_2$	105±48	11.8±4.6bc	24.0	11.24
	$MgSO_4$	56.0±13.1	8.2±1.5bc	16.7	14.64
200	control	509±292	40.5±18.4	100.0	7.96
	NaCl	74.5±20.9	11.0±2.2a	27.2	14.76
	Na_2SO_4	36.5±7.7	6.0±0.8b	14.8	16.44
	KCl	17.2±1.9	3.2±0.5cd	7.9	18.60

Table 4. (continued)

200 (cont'd)	K_2SO_4	14.8±3.4	2.0±0.0d	4.9	13.51
	$CaCl_2$	31.5±6.8	6.2±1.3b	21.7	27.94
	$MgCl_2$	23.2±6.0	4.8±1.0bc	11.9	20.69
	$MgSO_4$	25.0±5.3	5.0±0.8bc	12.3	20.00

* NaCl was present in the basic medium at a concentration of 10 meq/liter.

** Milliosmoles

\+ Means for each salt concentration followed by a common letter do not differ significantly at the 0.05 level. Control values were not included in the statistical analysis.

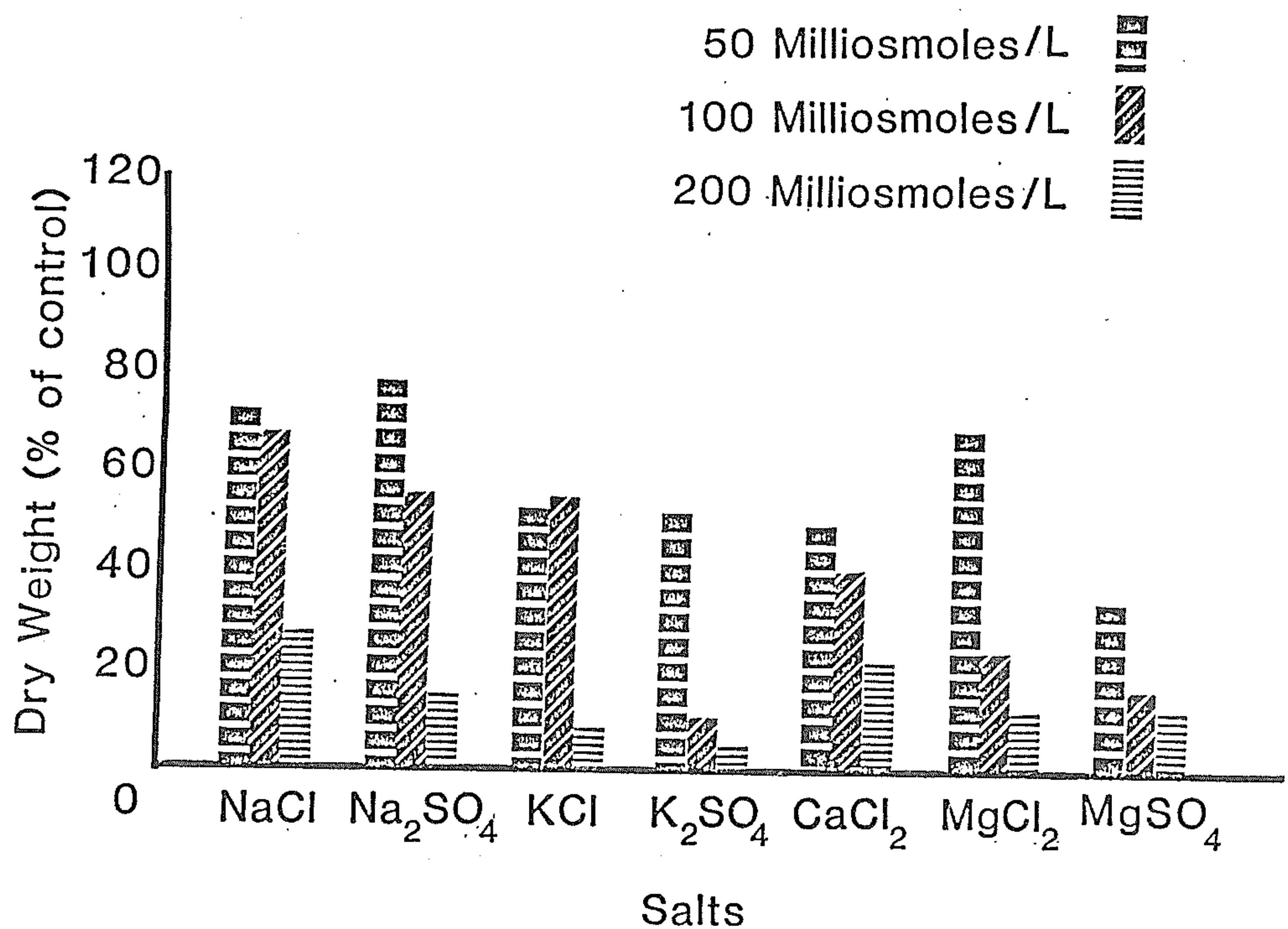

Figure 5. Effect of equal osmolar concentrations of various salts on growth of *A. mexicana*

Table 5.

Cation content of *Azolla mexicana* grown at several NaCl concentrations (N=4)

NaCl meq/liter	Tissue	Dry Wt mg	Fronds/Roots	Ca %	Mg %	Na %	K %	Fe ppm	Zn ppm	Mn ppm	Cu ppm
0	Fronds	40.2±6.4	2.83	0.23±0.01	0.246±0.020	0.57±0.04	5.33±0.05	382±29	64±7	20±2	27±3
0	Roots	14.2±1.7		0.57±0.02	0.156±0.013	0.65±0.04	6.89±0.31	1610±597	51±13	7.2±1.3	93±24
10	Fronds	42.8±8.8	3.34	0.22±0.01	0.230±0.012	2.91±0.08	2.72±0.14	345±19	59±4	21±2	25±2
10	Roots	12.8±3.1		0.58±0.05	0.103±0.012	2.61±0.17	5.52±0.56	992±236	39±7	8.2±1.7	80±8
50	Fronds	24.8±6.3	4.96	0.18±0.01	0.151±0.005	4.70±0.44	1.96±0.17	362±40	52±13	16±5	16±3
50	Roots	5.0±1.6		0.54±0.05	0.136±0.028	3.21±0.23	6.22±0.48	1312±327	38±10	7.2±5.2	57±14
100	Fronds	13.2±3.1	13.2	0.12±0.01	0.098±0.014	3.44±0.55	1.92±0.16	375±65	28±5	6.8±1.0	12±4
100	Roots*	1.0±0		0.77	0.222	2.67	6.13	6500	38	38	96
150	Fronds & Roots	6.5±1.7	--	0.15±0.05	0.090±0.022	2.17±0.42	2.38±0.13	1392±993	15±2**	5.5±3.8	19±8

* All samples combined for cation analysis.

** N=3

Table 6.

Chlorine content of *Azolla mexicana* grown at several NaCl concentrations (N=4)

NaCl meq/liter	Tissue	Dry Wt mg	Fronds/Roots	Cl %
0	Fronds*	21.2±4.7	4.24	1.47±0.12
0	Roots*	5.0±1.4		1.68±0.27
10	Fronds*	24.0±13.0	3.81	2.39±0.07
10	Roots*	6.3±3.1		3.05±0.28
50	Fronds	21.2±4.2	5.05	4.56±0.27
50	Roots	4.2±1.3		4.38±0.36
100	Fronds	7.5±2.6	6.25	3.22±0.61
100	Roots	1.2±0.5		3.12±0.82
150	Fronds & Roots	3.0±0.0	--	2.47±0.18

* N=3

Effects of NaCl Salinity on Chemical Composition of *Azolla*

The effects of various concentrations of NaCl on the inorganic composition of *Azolla* fronds and roots are presented in tables 5 and 6. A concentration of 100 meq/liter NaCl, which strongly inhibits total plant yield, is more damaging to growth of the small root system of *Azolla* than to the fronds when these organs are evaluated separately as indicated by the frond to root ratio.

As the NaCl concentration of the medium was increased from 0 to 10 meq/liter the Na concentration of tissue increased by a factor of 4 to 5 indicative of the low sodium concentration in the basic growth medium (table 5). Chlorine is an abundant constituent of the basic medium and increased by a factor of less than two with the addition of 10 meq/liter NaCl to the medium (table 6). Maximum concentrations of both Na and Cl were observed in fronds and roots of *Azolla* grown at 50 meq/liter NaCl. The K content of the fronds decreased substantially with addition of 10 and 50 meq/liter NaCl to the medium; however, the K content of the roots was essentially unaffected by increasing NaCl concentrations. As the NaCl concentration was increased to 50 meq/liter the Mg concentration in fronds decreased. The tissue content of Ca and the micronutrient cations were essentially unaffected in the range of 0 to 50 meq/liter NaCl. At 100 meq/liter NaCl, growth inhibition was severe and the frond contents of Ca, Mg, Zn, Mn, and Cu decreased. These effects of salinized growth medium on the inorganic composition of *Azolla* must be considered if *Azolla* is to be used as a compost material or as a feed in aquaculture systems or for livestock.

The amino acid proline often increases greatly in plant tissue during water or osmotic stress. Proline has been suggested to serve as a compatible osmotic solute that enables plant cells to lower their osmotic

Table 7.

The effect of artificial seawater (Instant Ocean) on growth of Azolla mexicana (N=4)

Instant Ocean %	EC*	Fresh Wt mg	Dry Wt mg	Relative Yield (%)	Dry Wt %
0	0.46	543±202	35.8±11.0ab**	100	6.59
2.50	1.79	694±185	41.8±10.4a	116.8	6.02
9.45	4.74	371±115	27.0±5.9b	75.4	7.28
20.30	9.25	65.0±19.3	9.5±3.1c	26.5	14.62
32.25	12.6	27.8±8.0	6.0±1.2c	16.8	21.58
42.2	15.5	19.2±4.6	5.0±1.6c	14.0	26.04

* Electrical conductivity in millimhos/cm

** Means followed by a common letter do not differ significantly at the 0.05 level.

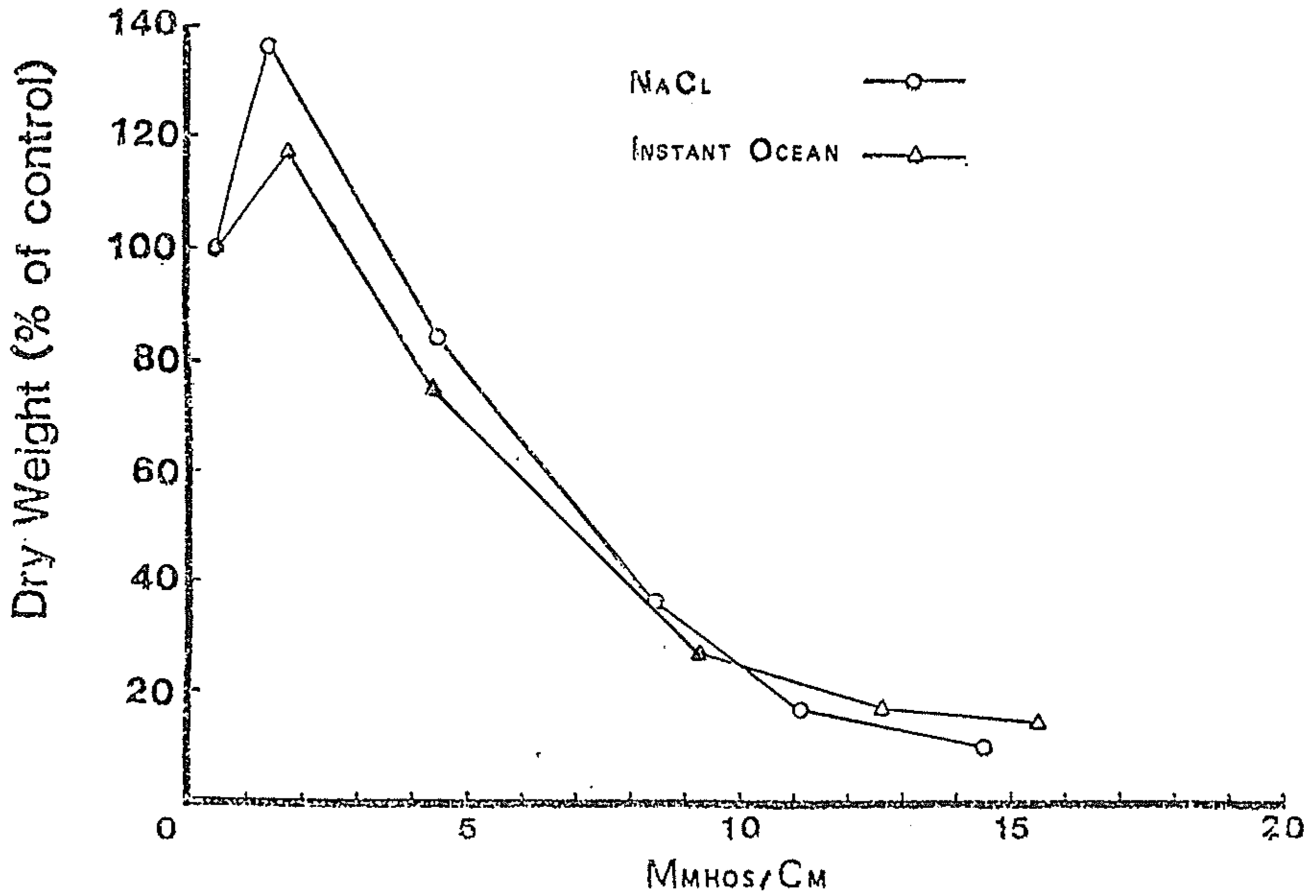

Figure 6. Effect of NaCl and Instant Ocean salinity on growth of *A. mexicana*

potential. The free proline concentration in non-stressed *A. mexicana* fronds and roots was respectively 0.04 and 0.03 μg/mg fresh weight and did not change appreciably with additions of up to 100 meq/liter NaCl to the growth medium.

Effects of Artificial Seawater on Growth of Azolla

Growth of *Azolla* was evaluated using artificial seawater (Instant Ocean, Aquarium Systems, Mentor, Ohio 44060 USA) to salinize the growth medium (table 7). The relative dry weight of *Azolla* grown at various concentrations of artificial seawater is compared to growth with added NaCl (data from table 1, experiment 2) as a function of the electrical conductivity of the medium (figure 6). Compared on the basis of electrical conductivity, growth inhibition by artificial seawater is very similar to growth inhibition by NaCl. The presence of other salts in artificial seawater neither alleviated nor increased the growth inhibiting effects of the dominant NaCl in seawater.

Effects of Polyethylene Glycol-6000 on Growth of Azolla

Growth of *Azolla* was severely inhibited by the non-ionic osmotic agent polyethylene glycol-6000 (PEG-6000) as indicated in table 8. When the relative yield is expressed as a function of osmotic potential of the added PEG-6000 or NaCl, it is apparent that PEG-6000 was much more inhibitory than a similar osmotic stress imposed by NaCl (figure 7).

Effects of NaCl Salinity on Growth of Azolla Supplied with Fixed Nitrogen

Experiments were conducted to determine the effect of added NaCl on the growth of *A. mexicana* when fixed nitrogen was supplied as nitrate (table 9). The relative yield as a percent of the control dry weight was significantly

Table 8.

The effect of polyethylene glycol-6000 (PEG-6000) induced osmotic stress on growth and nitrogen content of Azolla mexicana (N=4)

PEG g/liter	MPa*	Fresh Wt mg	Dry Wt mg	Relative Yield (%)	Dry Wt. %	Nitrogen %
0**	0	921±294	60.0±15.9a+	100	6.51	5.12±0.44
30	-0.04	428±97	43.5±12.9b	72.5	10.16	5.06±0.55
60	-0.075	278±58	28.8±6.2c	48.0	10.36	3.81±0.94
120**	-0.20	34.0±16.5	8.3±1.5d	13.8	24.41	2.02±0.21
152.5	-0.31	38.5±13.3	9.2±2.1d	15.3	23.90	1.86±0.12
177.5	-0.40	25.8±7.8	7.0±1.4d	11.7	27.13	2.01±0.34

* Osmotic potentials for PEG-6000 solutions at 23°C were obtained from unpublished data of Dr. B.E. Michel, Department of Botany, University of Georgia, Athens, GA, 30602.

** N=3

\+ Means followed by a common letter do not differ significantly at the 0.05 level.

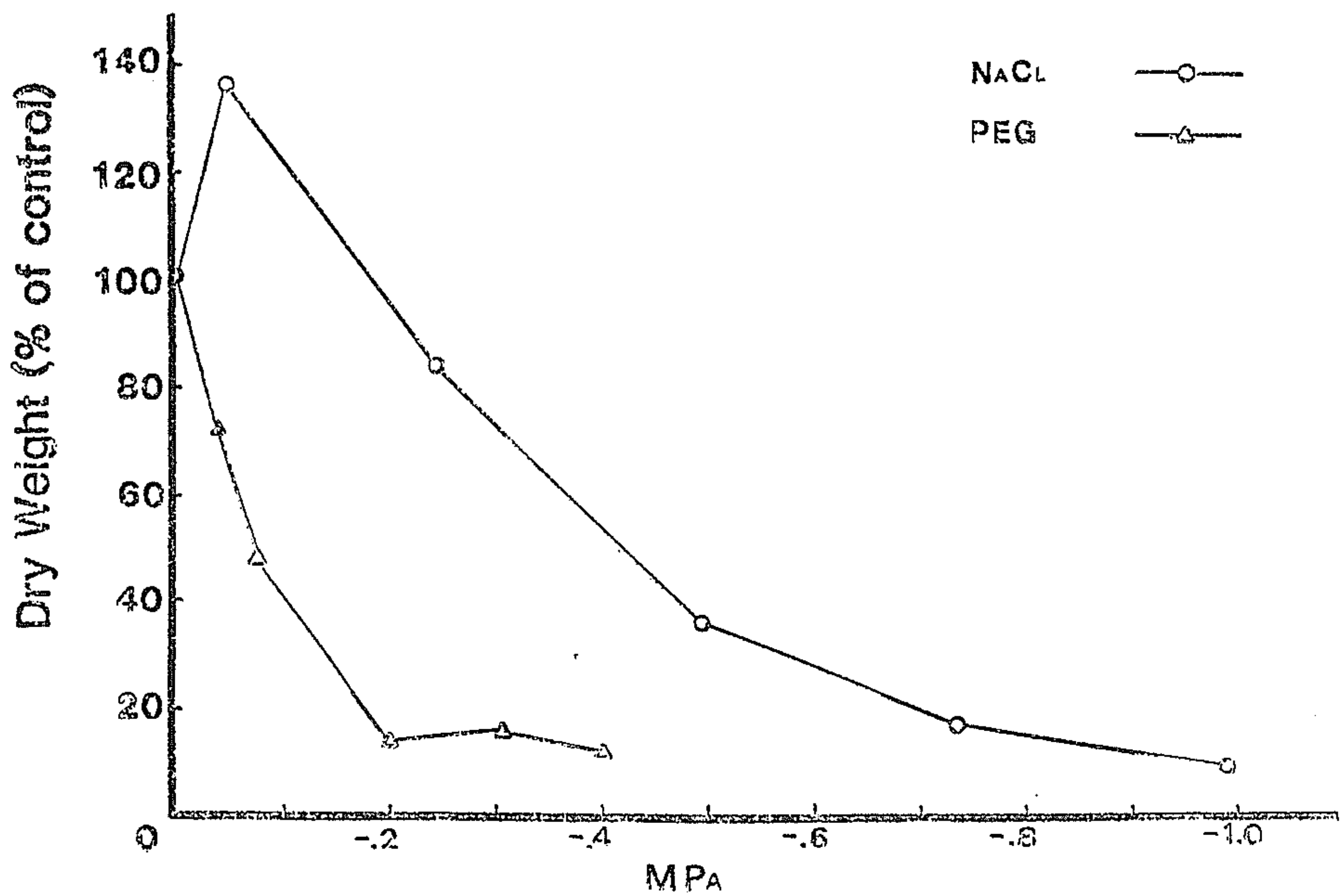

Figure 7. Effect of NaCl and polyethylene glycol-6000 on growth of _A. mexicana_

Table 9.

The effect of NaCl on growth and nitrogen content of <u>Azolla mexicana</u> on nitrogen containing medium (N=4)

	NaCl meq/liter	Fresh Wt mg	Dry Wt mg	Relative Yield (%)	Dry Wt %	Nitrogen %
EXP 1						
	0	318±52	16.2±1.5ab*	100.0	5.09	6.41±1.05
	10	442±53	20.5±2.4a	126.5	4.64	5.89±0.43
	50	368±69	19.5±5.7a	120.4	5.30	3.20±0.13
	100	232±68	17.0±2.4ab	104.9	7.33	2.92±0.88
	150	137±40	12.8±1.2b	79.0	9.34	2.63±0.53
	200	37.0±4.2	8.0±1.4c	49.4	21.62	1.77±0.16
	250	19.5±1.0	3.0±0.0d	18.5	15.38	1.91±0.30
EXP 2						
	0	320±78	19.2±4.7a	100.0	6.00	
	10	452±50	21.8±1.7a	113.5	4.82	
	50	365±84	19.2±3.6a	100.0	5.26	
	100	206±89	17.5±3.4a	91.1	8.50	
	150	56.2±9.5	10.8±1.0b	56.2	19.22	
	200	14.8±3.0	4.2±1.3c	21.9	28.38	
	250	14.2±2.8	3.2±1.0c	16.7	22.54	

* Means for each experiment followed by a common letter do not differ significantly at the 0.05 level.

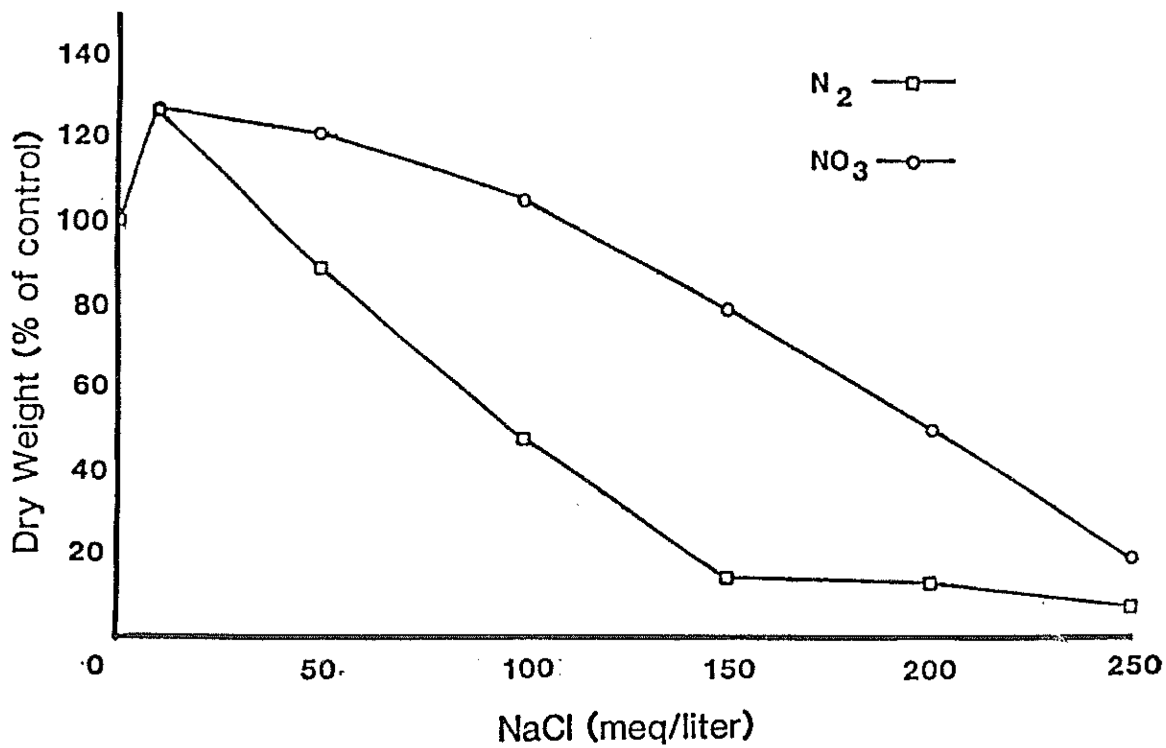

Figure 8. Effect of NaCl salinity on growth of _A. mexicana_ on nitrogen gas and nitrate

decreased only at NaCl concentrations of 150 meq/liter or greater. Thus when supplied with a source of fixed nitrogen Azolla was able to grow in NaCl concentrations (e.g. 100 meg/liter) that severely inhibited growth in the absence of fixed nitrogen (figure 8).

The Kjeldahl nitrogen content of the nitrate supplied Azolla decreased from 6.4 percent without added NaCl to 3.2 percent as the NaCl concentration was increased to 50 meq/liter. Further increases in NaCl resulted in a gradual decrease in the Kjeldahl nitrogen content.

Growth of Azolla supplied with nitrate was slower than under conditions of obligatory nitrogen fixation. Experiments with fixed nitrogen were conducted for 28 days instead of 16 days as in experiments using nitrogen-free medium; nevertheless, yields were much smaller in experiments with nitrate-nitrogen than when nitrogen-free medium was supplied. The use of smaller vessels and a smaller volume of medium in experiments with fixed nitrogen than in experiments with nitrogen-free medium probably contributed to the lower growth rates observed.

To determine if the low growth rate of Azolla supplied with nitrate-nitrogen was related to the requirement for nitrate reduction, *A. mexicana* was grown in medium with a constant amount of nitrogen supplied as nitrate, ammonium, or as various combinations of these two nitrogen sources. The data in table 10 indicate that yield of Azolla was not affected by variation in the proportions of nitrate- and ammonium-nitrogen supplied.

Effects of NaCl Salinity on Nitrogen Fixation Activity and Anabaena Abundance in Azolla

The effect of NaCl on nitrogen fixation (nitrogenase activity measured

Table 10.

The effect of nitrate and ammonium nitrogen sources on growth of *Azolla mexicana* (N=4)

$NO_3:NH_4$*	Fresh Wt mg	Dry Wt mg	Dry Wt %
4:0	402±112	20.7±4.6	5.15
3:1	366±66	19.8±3.4	5.41
2:2	324±96	17.5±5.3	5.40
1:3	338±65	19.0±4.2	5.62
0:4	322±62	19.8±3.9	6.15

* Nitrate:ammonium in milliequivalents/liter

Table 11.

The effect of NaCl on acetylene reduction activity (ARA) and *Anabaena* abundance when *Azolla mexicana* was grown on nitrogen-free and nitrate containing medium (N=3)

Medium		NaCl (meq/liter)						
		0	10	50	100	150	200	250
Nitrogen-free								
	ARA*	4312 ±416	3303 ±397	2978 ±659	3016 ±297	0	0	- -
Nitrate								
	Anabaena cells (%)**	100	99.4	85.1	80.4	14.7	18.5	20.5

* Acetylene reduction activity in nmoles/g fresh weight ± standard deviation

** Data are given as percentages of the control count/g fresh weight of fronds. The control tissue contained 6.85 x 10^6 cells/g.

by the acetylene reduction method) by _A. mexicana_ is reported in table 11. Nitrogenase activity decreased only slowly as the NaCl concentration was increased to 100 meq/liter; however, nitrogenase activity was not measureable in plants grown at NaCl concentrations of 150 meq/liter or higher. When the number of _Anabaena_ cells in fronds of nitrate-supplied _Azolla_ was determined, it was found that the _Anabaena_ cells decreased only slightly as the NaCl concentration increased from 0 to 100 meq/liter; however, the _Anabaena_ cells declined greatly as the NaCl concentration increased from 100 to 150 meq/liter (table 11). The yield of nitrate supplied _A. mexicana_, decreased only moderately to 68 percent of the control dry weight at 150 meq/liter (mean for two experiments in table 9). These results suggest that growth of the symbiotic _Anabaena_, nitrogen fixation, and/or interactive transport processes are more sensitive to salinity than growth of the host fern when supplied with fixed nitrogen. Thus if the salt tolerance of the microsymbiont, _Anabaena azollae_, can be increased, _Azolla_ should be able to grow satisfactorily in significantly more saline water without fixed nitrogen.

Effects of Calcium and Phosphorus Nutrition on Growth of Azolla in Saline Media

Increased Ca concentrations have been found to alleviate salt injury in agricultural plants (LaHaye and Epstein 1969). To test the effect of Ca nutrition on salt tolerance of _Azolla_, the effect of Ca concentration on yield of _Azolla_ was investigated at the growth inhibiting NaCl concentration of 100 meq/liter. In this experiment the basic nitrogen-free medium was prepared with KCl replacing $CaCl_2$ to maintain a constant Cl concentration and $CaSO_4$ was varied. When Ca was omitted severe growth inhibition occurred

Table 12.

The effect of calcium concentration on growth of <u>Azolla mexicana</u> at an inhibitory concentration of NaCl (N=4)*

NaCl meq/liter	$CaSO_4$ meq/liter	Fresh Wt mg	Dry Wt mg	Relative Yield (%)	% Dry Wt
0**	1.0	546±316	43.3±19.0	100	7.93
10**	1.0	561±304	38.7±18.1	89.4	6.90
100	0	8.8±1.3	2.5±0.6d+	5.8	28.41
100	0.25	99.2±39.4	11.2±3.3bc	25.9	11.29
100**	0.50	99.7±42.1	10.3±2.5bc	23.8	10.33
100	1.0	58.8±22.5	7.8±2.4c	18.0	13.27
100	5.0	151±71	14.5±5.1ab	33.5	9.60
100	10.0	171±36	16.8±2.9a	38.8	9.82
100	20.0	70.2±17.7	9.8±2.2bc	22.6	13.96

* In this experiment KCl replaced $CaCl_2$ in the basic medium thus maintaining a constant concentration of Cl while $CaSO_4$ was varied.

** N=3

\+ Means for 100 meq/liter NaCl treatments followed by a common letter do not differ significantly at the 0.05 level. Cultures grown at lower salt concentrations were not included in the statistical analysis.

Table 13.

The effect of phosphorus on growth of *Azolla mexicana* at various NaCl concentrations (N=3)

NaCl meq/liter	P mmoles/liter	Fresh Wt mg	Dry Wt mg	Relative* Yield (%)
0	0.25	790±243	52.3±12.0	100
0	0.75	857±140	52.3±6.7	100
10	0.25	972±353	59.7±20.0	114.1
10	0.75	958±199	58.3±13.0	111.5
50	0.25	596±252	41.3±14.2	79.0
50	0.75	374±114	22.3±3.1	42.6
100	0.25	135±63	15.3±5.7	29.3
100**	0.75	89.5±78.0	9.8±6.6	18.7
150**	0.25	28.5±18.3	5.0±2.8	9.6
150**	0.75	23.5±6.6	5.2±0.5	9.9
200**	0.25	17.8±1.3	3.5±0.6	6.7
200**	0.75	18.2±6.7	5.5±1.3	10.5

* Computed relative to 0.25 mmoles/liter P without added NaCl

** N=4

at 100 meq/liter NaCl (table 12). Addition of $CaSO_4$ at 0.25 meq/liter greatly increased yield of the fern, and there is a trend of additional growth increases up to 10 meq/liter $CaSO_4$; however, growth declined at 20 meq/liter $CaSO_4$. These results indicate that the $CaSO_4$ concentration of 2 meq/liter used in the basic nitrogen-free Azolla medium was not optimum for growth in salinized medium and yields can be enhanced by increasing the $CaSO_4$ concentration to 10 meq/liter.

Optimum P nutrition has been considered to reduce salt injury in agricultural plants; however, increasing P from 0.25 to 0.75 mmoles/liter did not decrease NaCl inhibition of Azolla growth (table 13). The phosphorus concentration of the basic Azolla medium was apparently adequate for maximum growth in the salinized solutions.

Selection for Salt Tolerance in Azolla

In an attempt to select salt tolerant individuals of Azolla, large populations of *A. mexicana* from the Corrales stock were grown in a greenhouse in trays containing the basic medium with addition of 130 meq/liter NaCl. This concentration had been demonstrated to be highly inhibitory to Azolla growth. After four months the few plants surviving this stress were collected and returned to the basic medium at 100 meq/liter NaCl. When growth of the selected plants resumed, their salt tolerance was evaluated in a growth chamber experiment at various concentrations of NaCl. Based on the data in table 14, the salt tolerance of the selected Azolla was not enhanced compared to the original stock (table 1). It appears likely that the few plants that survived the selection process at 130 meq/liter NaCl may have been utilizing nitrogen released from dead and decomposing plants and consequently the host and

Table 14.

The effect of NaCl on growth of *Azolla mexicana* selected on NaCl containing medium (N=2)

NaCl meq/liter	Fresh Wt mg	Dry Wt mg	Relative Yield (%)	% Dry Wt
0	467±99	30.0±8.5	100.0	6.42
10	464±323	31.0±21.2	103.3	6.68
50	204±33	15.0±0.0	50.0	7.35
100	98.0±67.9	10.0±5.7	33.3	10.20
150	19.0±2.8	3.8±0.4	12.7	20.00
200	16.0±1.4	3.0±0.5	10.0	18.75

Table 15.

The effect of NaCl on growth of <u>Azolla mexicana</u> under greenhouse conditions (N=2)*

NaCl meq/liter	Fresh Wt g	Dry Wt g	Relative Yield (%)	% Dry Wt
0	28.91±5.18	1.62±0.60	100	5.60
10	20.97±7.98	1.15±0.55	71.0	5.48
50	8.63±1.30	0.482±0.035	29.8	5.59
75	1.99±0.10	0.138±0.011	8.5	6.93
100	0.34±0.30	0.040±0.001	2.5	11.76

* Cultures were initiated by addition of 0.250 g (fresh weight) of <u>Azolla</u>; plants were harvested after 21 days of growth.

microsymbiont were not selected for salt tolerance under conditions of obligatory nitrogen fixation.

Evaluation of Azolla Growth in Salinized Medium Under Greenhouse Conditions

A greenhouse experiment was conducted to evaluate the growth of *A. mexicana* under more realistic mass culture conditions using trays containing 5 liters of the basic culture medium and various levels of NaCl salinity. Greater sensitivity to NaCl was observed in the greenhouse experiment (table 15) than in environmental chamber experiments (table 1). While light and temperature conditions differed in the two types of experiments, the greater sensitivity to salinity of plants grown in the greenhouse may be related to lower relative humidity and greater transpiration rates of *Azolla* grown in open trays compared to the covered beakers used in environmental chamber studies. The more rapid transpiration rates may have increased the concentration of Na and Cl in the fronds and hence enhanced the degree of salt damage.

CONCLUSIONS AND SUMMARY

Growth of *Azolla mexicana* collected near Corrales, New Mexico, was stimulated in nitrogen-free medium containing 10 meq/liter NaCl compared to controls without added NaCl. At 50 and 100 meq/liter NaCl growth was reduced to 86 percent and 42 percent of the control dry weight respectively. The nitrogen content of *Azolla* decreased from about 5 percent on a dry weight basis to 3.5 percent at 100 meq/liter NaCl. The electrical conductivity of medium containing 50 and 100 meq/liter NaCl was 4.5 and 8.5 mmhos/cm respectively. Water having an electrical conductivity exceeding 2.25 mmhos/cm is classified as very high salinity hazard irrigation water (USDA 1954). Therefore, the results of these environmental chamber studies indicate that *A. mexicana* can be grown in water that would not ordinarily be suitable for irrigation.

Several other species of *Azolla* as well as two cultures of *A. mexicana* collected from medium to high salinity hazard waters in the Rio Grande Valley were evaluated for growth in medium containing NaCl salinity. *Azolla caroliniana* from Madison, Wisconsin, exhibited a similar response to NaCl as *A. mexicana* from Corrales, New Mexico. *Azolla caroliniana* from North Carolina, *A. pinnata*, and *A. filiculoides* from Mono County, California, appeared less salt tolerant than *A. mexicana*. The cultures of *A. mexicana* from medium to high salinity hazard waters in the Rio Grande Valley were not more salt tolerant than *A. mexicana* from Corrales, New Mexico. These results suggest that genetic differences in salt tolerance may exist in different populations of *Azolla*. Because *Azolla* is readily dispersed by flowing waters and the *A. mexicana* populations were all collected within 100 miles of each other near the Rio Grande, it would not be surprising if they were very similar genetically. Further searches for *Azolla* ecotypes that are geographically isolated in moderately saline waters will probably provide ecotypes that have evolved enhanced salt tolerance.

Of the various salts studied, *Azolla* was more tolerant of Na salts compared to Ca, Mg and K salts. Sulfate salts tended to be more toxic than the corresponding Cl salts. These results are as expected because when non-halophytic plants have been exposed to salinity, NaCl has been the major salt in the vast majority of natural habitats. Hence salt tolerance has evolved primarily in response to NaCl salinity. *Azolla* was extremely sensitive to the non-ionic osmotic agent PEG-6000.

Azolla provided with fixed nitrogen as nitrate was able to grow at 100 meq/liter NaCl without significant depression of dry weight compared to the control without added NaCl. The number of *Anabaena* cells in fronds decreased by 5-fold as NaCl increased from 100 to 150 meq/liter while dry weight decreased by a factor of less than 2. Nitrogen fixation by *Azolla* growing in nitrogen-free medium decreased slowly as NaCl was increased to 100 meq/liter and was not detectable at 150 meq/liter. These results suggest that the host fern is more salt tolerant than the symbiotic *Anabaena*. A possible approach to enhancing salt tolerance of symbiotic *Azolla* may be through increasing the salt tolerance of isolated *Anabaena azollae* by selection for NaCl tolerant variants or possibly by genetic engineering methods. The salt tolerant *Anabaena azollae* then could be reintroduced into cyanobacteria-free *Azolla*.

The results of these environmental chamber studies demonstrate that *Azolla* can grow satisfactorily in water that is characterized as having a medium to high salinity hazard. It is recommended that this investigation be expanded to evaluate *Azolla* growth under hydroponics conditions in a greenhouse using naturally saline water with added plant nutrients other than nitrogen. A second important area for future research should be the evaluation of the applications of *Azolla* produced in saline water as a food material in aquaculture systems, as a food for farm animals, or as a compost material.

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