Salts and the Infectivity of *Romanomermis culicivorax*

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Abstract: The effects of common inorganic ions found in freshwater on the infectivity of *Romanomermis culicivorax* and the survival of its host, *Culex pipiens,* were tested. In general, the median lethal concentrations found for *R. culicivorax* were greater than the reported median concentrations of these ions in freshwater but less than the reported maximum natural concentrations. The ion toxicity for *R. culicivorax* (on a molar basis) increased in the following order: sodium < potassium < calcium; and chloride < carbonate = sulfate < nitrate < nitrite < phosphate. The larvae of *C. pipiens* were generally 20 to 75 times as tolerant of higher ion concentrations as were the preparasitic stages of *R. culicivorax.* *Key Words:* Median lethal concentration, ion toxicity, mermithid nematodes, mosquitoes, *Culex pipiens.*

The development of populations of pesticide-resistant mosquitoes and ecological and economic considerations related to the use of pesticides in aquatic environments have provided an impetus to the consideration of natural control. A vast array of mosquito pathogens are known (9), and the fungi and mermithid nematodes hold the most promise for natural control of mosquito populations. Currently, the mermithids appear more promising since Petersen (12) has mass-reared one species, *Romanomermis culicivorax* Ross and Smith, and has demonstrated its efficacy in mosquito control.

The ecology of *R. culicivorax* needs to be understood thoroughly for effective control of mosquito populations. Petersen (10, 11) has initiated studies of the effects of both density-dependent and density-independent factors on the biology of *R. culicivorax.* Temperature effects on the infectivity of *R. culicivorax* (2) were recently defined. Petersen and Willis (11) have found that natural infections of *R. culicivorax* did not occur in Louisiana waters with conductivities greater than 350 micromhos/cm and that *R. culicivorax* was not infective in sodium chloride solutions with concentrations greater than 0.04 M. Further development of *R. culicivorax* as a biological control agent requires determination of the tolerance limits of the infective stage to salinity derived from the various ions common in freshwater. The present investigation was designed to determine the effects of common ions found in freshwater on the infectivity of *R. culicivorax* for *Culex pipiens* L.

MATERIALS AND METHODS

A representative series of cations and anions common in freshwater were obtained by preparing solutions of 0.01 to 20 g of salt per liter distilled water from the following salts: $NaCl$, KCl, $CaCl_2$, Na_2CO_3, K_2CO_3, $NaNO_3$, KNO_3, $Ca(NO_3)_2$, $NaNO_2$, KNO_2, Na_2SO_4, K_2SO_4, NaH_2PO_4 KH_2PO_4, and $Ca(H_2PO_4)_2$. All salts were reagent grade. The pH and conductivity of each solution was measured before each experiment.

Romanomermis culicivorax was propagated in *Culex pipiens* after procedures of Petersen and Willis (12). Used as the host for *R. culicivorax* was an autogenous strain of *C. pipiens* designated by Barr as 24g.

All test solutions (125 ml) were placed in 200-ml polystyrene food containers, and 20 (±1) first-instar larvae of *C. pipiens* were added to each container (3 replications per salt concentration). Sixty (±9) infective larvae of *R. culicivorax,* obtained by procedures described earlier (2), were added to each container. The containers were cov-

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ered and placed in the dark at 27 C. After 24 h, the infective period was terminated by pouring the contents of each container into a sieve (180-μm openings). The mosquitoes were washed off the sieve, returned to clean containers with 125 ml water, fed, and left at 27 C for 10 days (2). Then the number of postparasitic nematodes and adult mosquitoes in each container was determined. The percentage of postparasites recovered was based on the calculated number of preparasitic larvae added (2).

Four experiments were carried out. Seven concentrations (20, 10, 2.5, 1.0, 0.5, 0.25, and 0.1 g/liter) of each of the 15 salts were used in the first three experiments. There were two groups in each experiment: one group tested the response of the mosquito larvae alone, and the other tested the response of the infective nematode larvae and mosquito larvae to the salts. The fourth experiment involved 10 concentrations of each salt (10, 5, 2.5, 1.0, 0.5, 0.25, 0.1, 0.05, 0.025 and 0.01 g/liter). Three experimental groups were studied: the first two were as in the first three experiments, and the third group tested the infectivity of nematode larvae preexposed to the salts for 4 h before the mosquito larvae were placed in the infection containers.

The data from the four experiments were pooled, and median lethal concentrations of the salts for mosquito larvae and infective nematode larvae were calculated by probit analysis (3, 4, 5).

RESULTS

The salt effects were compared first on the basis of the maximum salt concentrations at which some mosquito survival and nematode infections occurred.

The salts least toxic to the mosquitoes were sodium sulfate and potassium sulfate, in which some survival occurred even at 20,000 mg/liter (Table 1). The most toxic salts to the mosquitoes were sodium nitrite and potassium nitrite, in which there was no survival above 1,000 mg/liter. Mosquitoes survived over a pH range of 3.4 to 10.5. The maximum conductivity allowing some mosquito survival was 20,000 micromhos per cm.

Nematodes retained infectivity, albeit at low levels, in 7 salts: sodium chloride, calcium chloride, sodium nitrate, potassium nitrate, calcium nitrate, potassium sulfate, and potassium phosphate, to concentrations of 2,500 mg/liter (Table 2). Infectivity was retained over a pH range of 3.6 to 8.6. The highest conductivity at which infection was achieved was 4,000 micromhos/cm.

A comparison of the maximum levels of mosquito survival (Table 1) and nematode infection (Table 2) showed that in all but two salts (potassium nitrite and potassium nitrate) the mosquitoes were more salt-tolerant than the nematodes (Fig. 1).

When the maximum levels at which infection occurred with mosquitoes and nematodes added simultaneously to the salt

TABLE 1. Maximum salt tolerances of the first-instar larvae of *Culex pipiens*.[a]

Salt	Concentration mg/liter	Concentration mM	pH	Conductivity (micromhos/cm)	Percent survival
$NaCl$	10,000	171	5.9	16,000	40
$CaCl_2$	10,000	90	6.3	12,200	32
KCl	10,000	134	6.2	16,000	23
Na_2CO_3	5,000	60	10.5	6,800	5
K_2CO_3	2,500	24	10.3	3,550	3
Na_2SO_4	20,000	141	6.0	18,000	2
K_2SO_4	20,000	115	5.9	20,000	5
$NaNO_3$	5,000	59	5.6	5,750	10
$Ca(NO_3)_2$	10,000	610	5.9	8,000	3
KNO_3	2,500	25	5.4	3,050	13
$NaNO_2$	1,000	15	6.1	1,525	2
KNO_2	1,000	12	6.5	1,520	8
NaH_2PO_4	10,000	83	4.6	5,200	18
$Ca(H_2PO_4)_2$	2,500	11	3.4	1,220	2
KH_2PO_4	10,000	74	4.5	6,100	2

[a]The maximum salt concentrations at which survival of mosquito larvae occurred.

TABLE 2. Maximum salinity tolerances of the preparasitic larvae of *Romanomermis culicivorax*.[a]

Salt	Concentration mg/liter	Concentration mM	pH	Conductivity (micromhos/cm)	Percent survival
NaCl	2,500	43	5.5	4,400	1
$CaCl_2$	2,500	23	5.8	3,460	1
KCl	500	7	5.4	730	5
Na_2CO_3	250	3	8.6	385	1
K_2CO_3	250	2	8.5	370	1
$NaNO_3$	2,500	28	5.4	3,030	1
$Ca(NO_3)_2$	2,500	15	5.7	1,825	1
KNO_3	2,500	25	5.4	3,050	1
$NaNO_2$	500	7	6.4	635	1
KNO_2	1,000	12	6.5	1,520	1
Na_2SO_4	500	4	5.5	620	7
K_2SO_4	2,500	14	5.5	3,300	1
NaH_2PO_4	1,000	8	4.7	620	1
$Ca(H_2PO_4)_2$	1,000	4	3.6	630	1
KH_2PO_4	2,500	18	4.5	1,675	3

[a]The maximum salt concentrations at which infectivity occurred.

solutions were compared with the maximum infection levels resulting when mosquitoes were not added until the nematodes had been exposed to the salt solutions for 4 h, no change was found in the maximum concentration at which some infection occurred for seven of the salts tested (Fig. 2). Four hours of previous exposure substantially reduced the maximum concentration at which the nematodes were infective in the remaining eight salts [NaCl, $CaCl_2$, $Ca(NO_3)_2$, $NaNO_2$, K_2SO_4, NaH_2PO_4, $Ca(H_2PO_4)_2$, and KH_2PO_4].

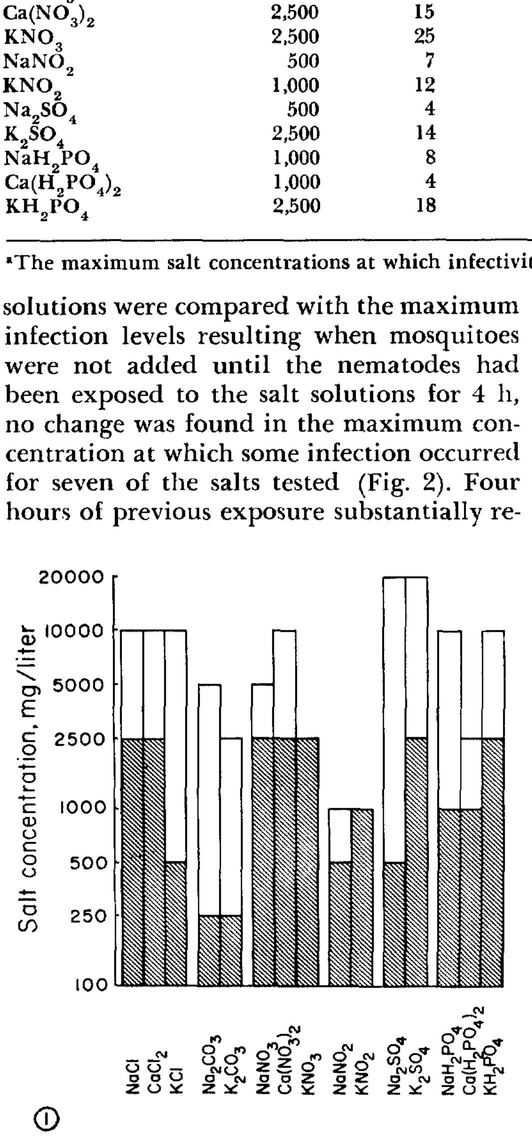

FIG. 1. Comparison of the maximum salt concentrations at which mosquitoes survived and nematodes were infective. Concentrations for mosquitoes and nematodes are equal for KNO_3 and KNO_2. Open bars, mosquito survival; cross hatch, nematode infectivity; concentration plotted on a logarithmic scale.

Subsequent data analysis was based on the individual ions in each salt experiment, so that the effects of the counterions could be determined (Table 3, 4, Fig. 2-10). All calculations were on a mg/liter basis for each ion, so that average concentrations for maximum tolerance and median lethal concentrations could be calculated for each series of counterions, e.g., sodium cation in the presence of the 6 anions tested.

The average maximum ion tolerances of the mosquito larvae were calculated (Table 3, 4) but it was necessary to recalculate the concentration of each ion as a molarity to compare the average effects of each ion within its ion class. The maximum tolerance for cations in order of decreasing tolerance was: sodium, 86 mM; potassium, 63 mM; and calcium, 54 mM. In the median-lethal-concentration series, however, the order of calcium and potassium changed: sodium, 50 mM; calcium, 19 mM; and potassium, 15 mM. The maximum tolerance for anions in order of decreasing tolerance was: chloride, 132 mM; sulfate, 128 mM; phosphate, 56 mM; nitrate, 36 mM; carbonate, 33 mM; and nitrite, 13 mM. The order of carbonate and nitrite changed in the median-lethal-concentration series: chloride, 112 mM; sulfate, 54 mM;

TABLE 3. Maximum cation tolerance and median lethal concentrations for the first-instar larvae of *Culex pipiens*.

Cation	Anion present	Maximum conc. tolerated (mg/liter)	LC_{50} (mg/liter)	95% CL[a] (mg/liter)
Na^+	Cl^-	3,930	3,230	1,832-16,466
	$CO_3^=$	1,085	133	58-261
	NO_3^-	1,355	55	14-109
	NO_2^-	333	10	2-20
	$SO_4^=$	3,240	1,244	788-2,463
	$H_2PO_4^-$	1,920	572	0-9,717
	mean	1,977	1,157	669-4,538
Ca^{++}	Cl^-	3,600	2,129	633-20,000+
	NO_3^-	2,400	69	1-217
	$H_2PO_4^-$	428	29	11-50
	mean	2,156	742	215-20,000+
K^+	Cl^-	5,240	2,692	896-20,000+
	$CO_3^=$	708	49	15-91
	NO_3^-	968	145	74-238
	NO_2^-	459	35	3-76
	$SO_4^=$	4,490	501	210-998
	$H_2PO_4^-$	2,870	41	0-161
	mean	2,456	644	200-20,000+

[a]Confidence limits for median lethal concentrations.

TABLE 4. Maximum anion tolerance and median lethal concentrations for the first-instar larvae of *Culex pipiens*.

Anion	Cation	Maximum conc. tolerated (mg/liter)	LC_{50} (mg/liter)	95% CL[a] (mg/liter)
Cl^-	Na^+	6,070	4,907	2,782-20,000+
	Ca^{++}	3,200	1,893	563-20,000+
	K^+	4,760	2,445	814-20,000+
	mean	4,677	3,984	2,089-20,000+
$CO_3^=$	Na^+	2,830	348	152-680
	K^+	1,085	49	15-91
	mean	1,958	212	84-386
NO_3^-	Na^+	3,645	147	38-293
	Ca^{++}	3,780	107	1-336
	K^+	1,533	230	117-377
	mean	2,986	161	52-335
NO_2^-	Na^+	666	20	4-40
	K^+	541	41	3-90
	mean	604	31	4-65
$SO_4^=$	Na^+	13,520	5,192	3,247-10,278
	K^+	11,020	198	83-395
	mean	12,270	2,695	1,665-5,337
$H_2PO_4^-$	Na^+	8,080	2,407	0-20,000+
	Ca^{++}	1,036	71	26-120
	K^+	7,170	103	0-402
	mean	5,429	860	9-13,805

[a]Confidence limits for median lethal concentrations.

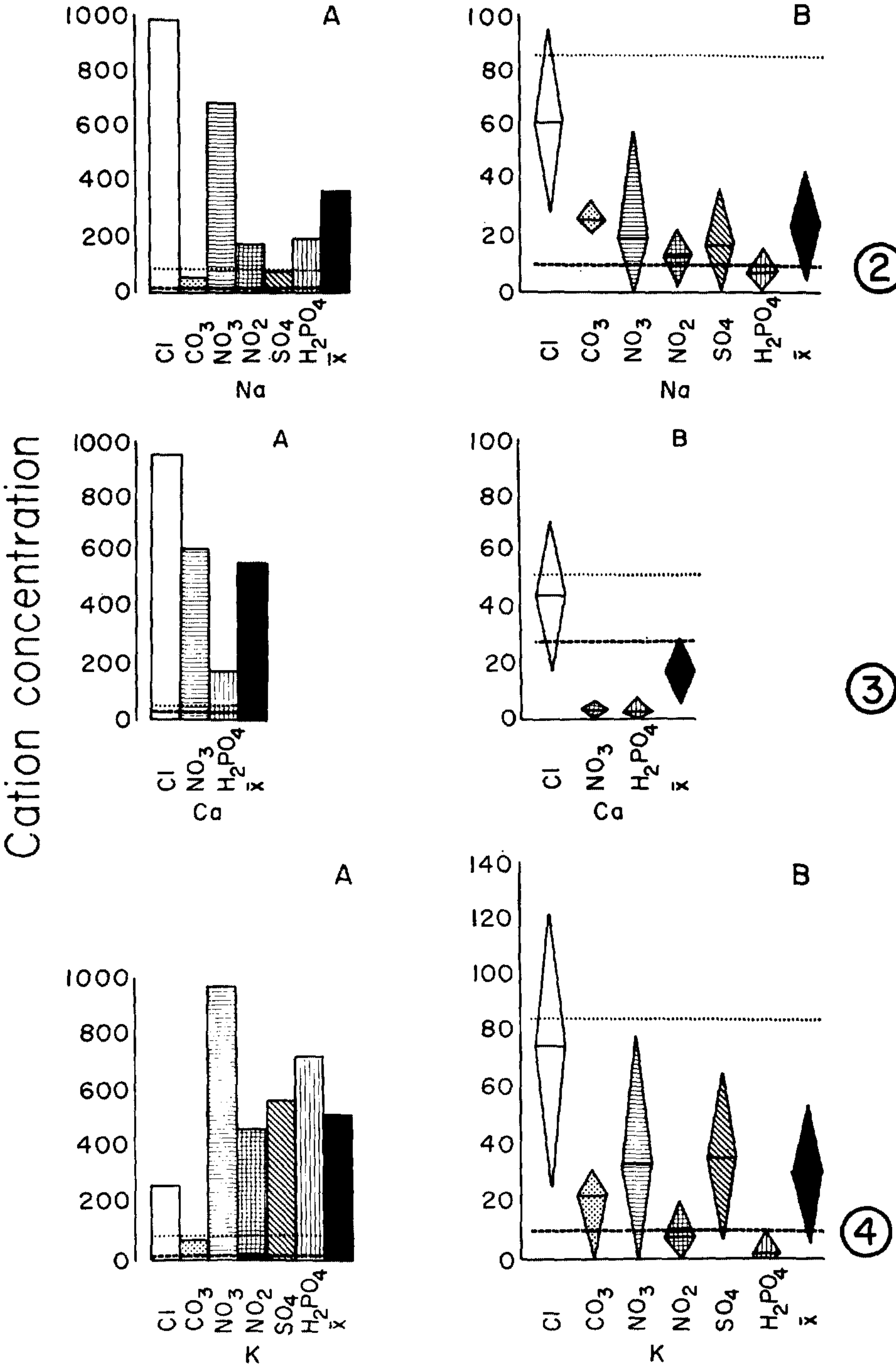

FIG. 2-4. Tolerance of *Romanomermis culicivorax* to cation concentrations (mg/liter ± 95% confidence limits). Cation concentrations in freshwater, median (— — —) and maximum (. . . .). *2)* Sodium. *3)* Calcium. *4)* Potassium. A) is the maximum tolerance, and B) is the median lethal concentration.

phosphate, 8.9 mM; carbonate, 3.5 mM; nitrate, 2.1 mM; and nitrite, 0.67 mM.

The toxicity of cations for the infective larvae of *R. culicivorax* was altered greatly by the anion present. Cation toxicity was lowest in the presence of chloride anions, and the toxicity was greatly increased in the presence of phosphate or nitrite anions (Fig. 2-5, 8, 10). Comparison of the cation effects in Fig. 2-4 showed the same relative effects of the anions with each cation. In general, the median lethal concentrations for the infective larvae of the cations and anions studied were greater than their levels as reported for freshwaters of North America, though less than the maximum concentrations found (16) (Fig. 2-4, 9).

The maximum tolerated concentrations of cations for the infective larvae were similar: sodium, 16 mM; calcium, 14 mM; and potassium, 13 mM. A different cation order existed, however, in the median-lethal-concentration series: sodium, 1.04 mM; potassium, 0.74 mM; and calcium, 0.42 mM.

The maximum tolerated concentrations of anions for the infective larvae were, in order of increasing toxicity: chloride, 24 mM; nitrate, 18 mM; phosphate, 10 mM; nitrite, 9.5 mM; sulfate, 8.9 mM; and carbonate, 2.1 mM. On the basis of median lethal concentrations, however, a substantial reordering occurred in calculation of the anion order: chloride, 1.9 mM; carbonate, 0.85 mM; sulfate, 0.81 mM; nitrate, 0.44 mM; nitrite, 0.37 mM; and phosphate, 0.12 mM.

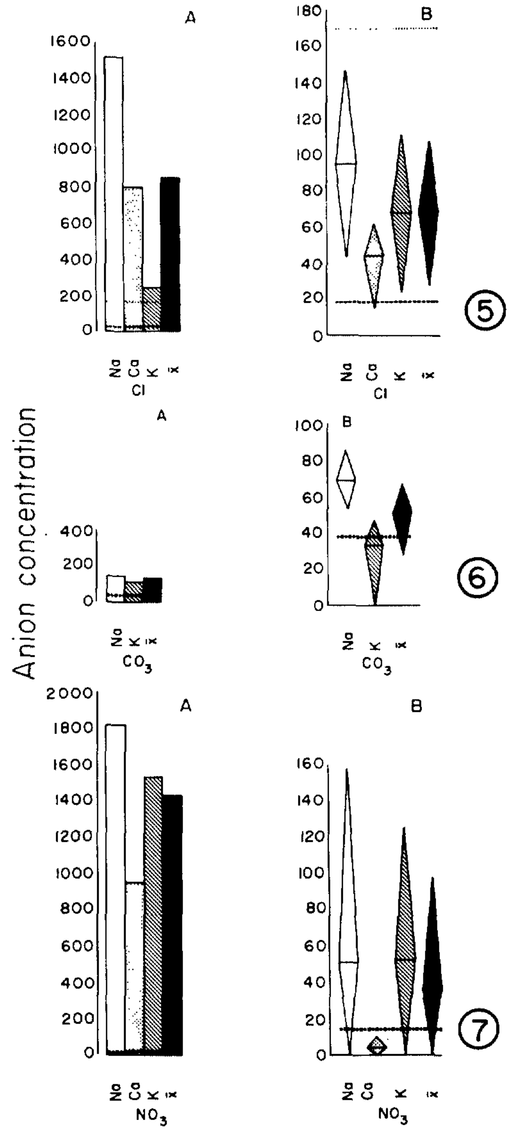

FIG. 5-7. Tolerance of *Romanomermis culicivorax* to anion concentrations (mg/liter ± 95% confidence limits). *5)* Chloride; concentrations in freshwater, median (— — —) and maximum (. . . .). *6)* Carbonate; typical concentration in freshwater (— — —). *7)* Nitrate; typical nitrate concentration in freshwater (— — —). A and B as in Fig. 2-4.

DISCUSSION

In this investigation, salt tolerances of the preparasites were determined by simultaneous exposure of both preparasites and hosts to various salt concentrations for 24 h. That procedure appeared to be a satisfactory simulation of field releases of preparasitic nematodes into freshwater of varying salt content since the majority of the preparasites of *R. culicivorax* penetrate the available mosquito hosts within 24 h (2, 10) under laboratory conditions and in field studies.

In general, the first-instar larvae of *C. pipiens* were more tolerant of the salts tested than were the infective larvae of *R. culicivorax*. Comparison of the median lethal concentrations for mosquito and nematode larvae showed that, for most cations and anions, the mosquito larvae tolerated ion concentrations 20 to 74 times those tolerated by the nematode larvae. The respective average concentrations of nitrite, nitrate, and carbonate tolerated, however, were only 2, 4, and 5 times those tolerated by the nematode larvae. With nitrite in the presence of potassium, the median lethal concentration for the nematode larvae showed a combined deleterious effect on both the infective parasite and the host. Further determination of the median lethal concentration of nitrite in the presence of potassium for the infective larvae would require a host with greater tolerance for this salt.

The median concentrations (mg/liter) for the ions found in 90% of the freshwaters in North America are: sodium and potassium combined, 10; calcium, 9; and sulfate, 32 (16). The maximum concentrations (mg/liter) found for those ions are: sodium and potassium combined, 85; calcium, 52; chloride, 170; and sulfate, 90 (6, 16). A typical lake may contain carbonate at 38 mg/liter, whereas 1 mg/liter is typical for spring water. Nitrate is found in low concentrations (1 mg/liter) in normal groundwater, whereas many water supplies contain 14 mg/liter and feedlot runoff may contain 83 mg/liter. Nitrite concentrations are not listed in the literature for natural waters but are always lower than the nitrate concentrations in the water source (6, 16). Normal phosphate concentrations are 1 mg/liter, whereas wastewater from fertilizer plants can contain as much as 30 mg/liter (6). The median lethal concentrations obtained for *R. culicivorax* are generally greater than the median concentrations of the common ions in freshwater of North America, indicating that *R. culicivorax* can be used successfully in most freshwater in North America. Elevated calcium concentrations and nitrite levels, however, would restrict its use. Elevated phosphate levels would be detrimental but would probably occur only in unusual situations, e.g., effluents from fertilizer plants.

Several investigators have studied the effects of salts on nematode survival, motility, and metabolism. Walker (15) reported that populations of *Pratylenchus penetrans* decreased in soil following the addition of nitrate, nitrite, organic nitrogen, and ammonium compounds. Nitrate was the least nematicidal, and nitrite the most. Similarly, nitrite inhibited the infectivity of *R. culicivorax* more than did nitrate. Stephenson (13) found that increasing salt

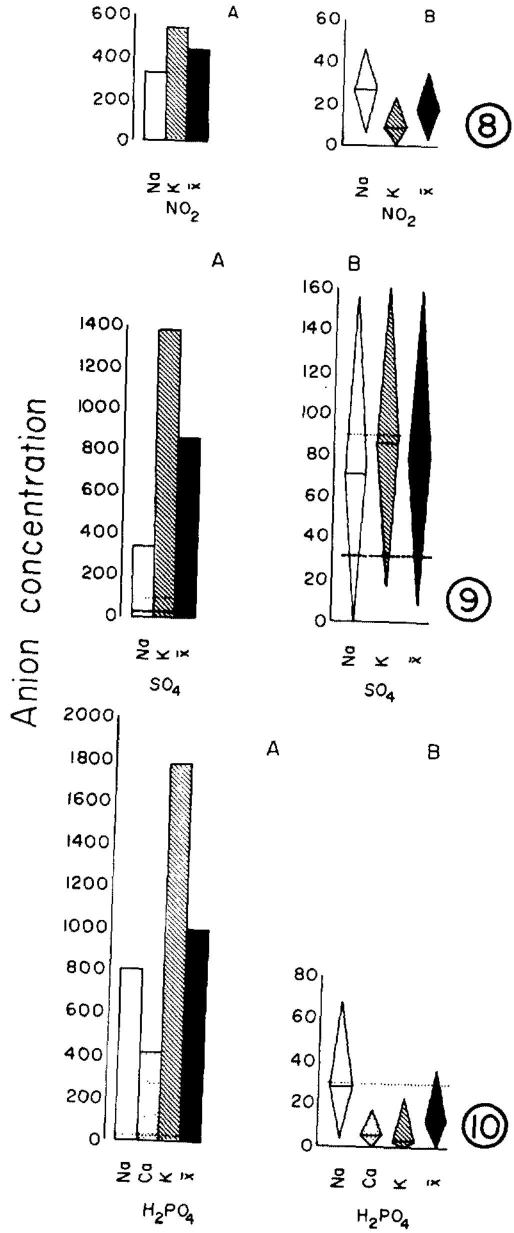

FIG. 8-10. Tolerance of *Romanomermis culicivorax* to anion concentrations (mg/liter ± 95% confidence limits). *8)* Nitrite. *9)* Sulfate; maximum (. . . .) and median (— — —) concentrations of sulfate in freshwater. *10)* Phospate; maximum concentration in freshwater (. . . .). A and B as in Fig. 2-4.

concentration caused body shrinkage and cessation of movement in *Rhabditis terrestris,* and the rates of penetration could be arranged as follows: KCl, $MgCl_2 < CaCl_2 < NaCl$. Other investigations of salt effects have been carried out on animal-parasitic nematodes. Von Brand (15) studied the effects of salts on the oxygen consumption of *Eustrongylides ignotus,* a nematode parasite of fish. Of the salts tested, only $NaNO_2$ (and KCl to a lesser degree) were toxic. Interestingly, the oxygen consumption of *E. ignotus* was stimulated by various ions according to the following series: cations – sodium = magnesium < calcium = ammonium < potassium; anions – chloride = sulfate < nitrite = nitrate < phosphate. That series is similar to series found for *R. culicivorax.* Bueding (1) found that high concentrations of KCl and $CaCl_2$ markedly decreased the metabolic activity and motility of *Litosomoides carinii,* a filarioid from cotton rats.

Most information available on the salt tolerances of freshwater animals deals with arthropods and vertebrates. The salt tolerances of a free-living turbellarian, *Polycelis nigra,* have been investigated, and the order of cation and anion toxicities was similar to the averages reported for *R. culicivorax* (7, 8). Although Jones (7, 8) found cations more toxic than anions, the average toxicity of the common freshwater anions and cations was equivalent for the infective stage of *R. culicivorax.*

The mechanism by which the ions affected the infectivity of *R. culicivorax* was not determined. The toxicity at elevated ion concentrations may have resulted from: 1) increased osmotic pressure and concomitant water loss from the nematodes; 2) imbalance in internal ion balance; 3) general toxic effects of nitrates and nitrites; and 4) altered internal acid-base balance. It may be speculated that, as with *E. ignotus* (14), an increase in metabolic rate stimulated by salts may have exhausted the energy resources of the infective stages before the host was contacted, thereby reducing the infective potential of the nematode. In addition, the decreased body water of nematodes at high salt concentrations would probably decrease their motility and, thereby, infectivity. At the salt concentrations obtained for median lethal concentrations, however, the most likely explanation is that ion imbalances occurred in the infective nematodes and that the ensuing metabolic disruptions interfered with the infective ability of the preparasites. Those questions will be the subject of future investigations.

Actual field situations do not have waters containing only single cations or anions, and further work approximating actual field conditions is necessary to determine the effects of combinations of ions on the infective ability of *R. culicivorax.* Another interesting field for future study is the effects of salts on other life-cycle stages to determine the effects of salinity on the ability of *R. culicivorax* to reproduce on a continuing basis in a body of water after preliminary applications of preparasites. Preliminary studies in this area indicate that adult nematodes are more salt-tolerant than are preparasites.

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