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MICROINVERTEBRATES IN CELSS HYDROPONIC RHIZOSPHERE: EXPERIMENTAL INVASION AS A TEST OF COMMUNITY STABILITY AND A TEST OF A METHOD TO MEASURE BACTERIVORY

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ABSTRACTS

The following report consists of two separate draft manuscripts, each prepared for submittal to a peer-reviewed journal after KSC colleague editorial review and final revisions. Manuscripts were prepared in journal-specific format. References for the 2 papers have been combined for this report. The abstracts of the two manuscripts are as follows:

1. Experimental invasion of aquatic rhizosphere habitat and invertebrate communities. Jenkins, D.G. and J.L. Garland. To be submitted to Oecologia.

Invasion of communities or ecosystems may be considered a disturbance, by which the stability of a community may be judged and the mechanisms regulating community composition inferred. We experimentally invaded aquatic rhizospheres of hydroponically-grown plants with three sets of epiphytic communities (invaders). Invaded rhizospheres were either sterile or pre-inoculated with organisms that normally inhabit the rhizospheres (residents). About 1/2 of invaders were successful with or without residents present. Few invader species were clearly excluded by the presence of residents, and invasion did not displace residents. Environmental conditions partially excluded invaders and biotic interactions were far less important in determining community composition after invasion.

2. Lysozyme analysis (Gonzalez et al. 1993 and Vrba et al. 1993) is neither protistan- or bacterivore-specific. Jenkins, D.G., C. Atkinson and J.L. Garland. To be submitted to Microbial Ecology.

Enzymatic assays of protistan bacterivory have been proposed recently (Gonzalez et al 1993 and Vrba et al. 1993). Both methods rely on lysozyme hydrolysis of the β(1-4) glycosidic bond between N-acetylglucosamine and the fluorogenic compound 4-methylumbelliferyl. We evaluated the specificity of the Gonzalez et al. method (acid lysozyme) to protistan bacterivory, and found that 5 of 6 protists and 20 of 21 (95%) metazoan genera tested had acid lysozyme. Therefore, the methods of Gonzalez et al. (1993), and by inference Vrba et al. (1993), are not protistan-specific. Lysozymes (e.g., acid lysozyme, β-N-acetylglucosaminidase) hydrolyze both peptidoglycan and chitin, meaning that bacterivory cannot be distinguished from chitin digestion by lysozyme analysis. Therefore, the methods of Gonzalez et al. (1993), and by inference Vrba et al. (1993), are not bacterivore-specific. Consequently, lysozyme-based analyses of bacterivory can not be applied reliably to samples more complex than isolated protistans that have been demonstrated to not also digest chitin, as some predatory protistans may.

EXPERIMENTAL INVASION OF AQUATIC RHIZOSPHERE HABITAT AND INVERTEBRATE COMMUNITIES

1.1 INTRODUCTION

"Invasion" and "colonization" may be used synonymously, but studies of invasion usually focus on the success of introduced, sometimes exotic species, the effects of those species on resident communities, or the traits of a community that make it invasible (Lodge 1993, Robinson and Dickerson 1984, Crawley 1987). Colonization studies tend to focus on timing of arrival, distances from source populations, species richness, and extinction, usually related to MacArthur and Wilson's island biogeography theory (MacArthur and Wilson 1967). Robinson and Dickerson (1984) characterized invasion theories in two ways: (1) invasibility is independent of community composition and implies nothing about community stability (e.g., MacArthur and Wilson 1967); or (2) invasibility depends on community composition, because composition affects stability, niche availability and niche overlap (e.g., Elton 1958, May 1974). The link between complexity and stability is largely intuitive and has long been the subject of an unresolved debate (May 1973), but invasibility has been used as a test of community niche availability, complexity, and stability (Crawley 1987, Robinson and Dickerson 1984, Lodge 1983).

In the long run, community composition is the net result of regional-scale processes (dispersal) and local-scale processes, such as competition and predation (Ricklefs 1987), and communities will always change as a result of the interplay between these ongoing processes. In the short run, we desire certain communities to be stable and predictable: organisms inhabiting the rhizosphere of hydroponically-grown plants at Kennedy Space Center, FL (KSC) is one of these communities. Various crops (e.g., wheat, potato) are grown hydroponically under carefully-regulated conditions during development and testing of a Controlled Ecological Life Support System (CELSS) for long-term space missions (Wheeler et al. 1996). Long-term dependence on life support functions (gas and water processing, food) in space requires that systems be stable. Microbial communities in hydroponic rhizosphere are considered stable (Strayer 1994), and invertebrate community composition has been limited and repeatable (Jenkins 1995, unpublished results). However, stability is best measured by a system's response to change.

We experimentally introduced three sets of epiphytic communities from natural submerged aquatic vegetation into the rhizosphere of hydroponic plants. If simultaneous invasion by multiple species can be considered a disturbance to the resident community, then this experiment was a test of that resident community's stability in response to that disturbance. We inferred the effects of residents on the invasion process by comparing invasion in habitat with and without residents.

Three possible mechanisms may limit resident community composition: 1) Environmental conditions. Hydroponic plants are grown in a nutrient medium intended to promote plant growth, but that may inhibit growth of some aquatic invertebrates. In addition, rhizospheres are kept dark to inhibit algal growth: should aquatic invertebrates require algae for food and not be be able to subsist on bacteria, they may be excluded. 2) Biotic interactions. Invertebrates that exist in the rhizospheres may exclude subsequent species via competition and predation. 3) Dispersal. Hydroponic rhizospheres are in environmental chambers or the Biomass Production Chamber (BPC; Wheeler et al. 1996), all of which are housed indoors. Little opportunity likely exists for aquatic invertebrates to arrive.

Specific questions addressed in this study were: (1) Can "invaders" successfully colonize hydroponic systems that lack resident organisms? If so, then the environment is not exclusive, and dispersal would be indicated as limiting community composition. If not, environmental conditions would be indicated as limiting community composition. (2) Can invaders successfully colonize hydroponic systems that contain residents? If so, biotic interactions would not be indicated as excluding invaders. If not, and if invaders can succeed in sterile hydroponic systems, then biotic interactions would be indicated as limiting community composition.

1.2 MATERIALS AND METHODS

The experiment consisted of two treatments (resident organism presence/absence and invader inoculum source), organized in randomized blocks. Each block contained one replicate of each treatment combination. Blocks were used to account for a potential effect of position relative to the mist source, which was anticipated to affect plant growth and could serve as a micro-invertebrate contamination source. Therefore, experimental units were arranged in four blocks, from right to left, with one replicate of each treatment combination in each block.

Wheat plants (*Triticum aestivum* L., cv. Yecoro rojo) were grown hydroponically in jugs within an environmental control chamber, which permitted regulation of light and humidity. Wheat is commonly used in CELSS experiments; it grows quickly in hydroponic medium and provides abundant rhizosphere for aquatic organisms within several weeks. Humidity was provided as deionized water mist, blown from the right end of the chamber (Figure 1). Relative humidity cycled between 56% (mist off) and 90% (mist on), and light intensity ranged from 125 µE at 25 cm elevation (jug top) to 363 µE at 80 cm elevation (approximate plant height at experiment end).

All seeds and materials were sterilized and handled aseptically to minimize contamination by non-experimental organisms. Each of the 24 2.2-L Nalgene wide-mouth jugs contained one wheat plant, growing hydroponically in filter-sterilized (0.2 µm) ½-strength Hoagland's solution (Morales 1995). Seedlings were 5 days old at experiment start-up, and were inserted in a split polyurethane foam plug between two strips of 83-um mesh Nitex mesh (to act as a wick for hydroponic medium until roots grew longer). The plant and foam plug were then inserted in a hole at the center of the jug lid, and the lid was placed on the jug. Each jug was wrapped in black polypropylene to inhibit algal growth.

Each lid had two other holes, stoppered with rubber septa: one served as an aeration port, the other as a sampling port. Facility air was passed through a liquid trap and then a 0.2-µm filter to a syringe needle, which was inserted through an aeration septum. A Pasteur pipette fitted into the bottom of the septum passed air to the bottom of the container. Hydroponic medium samples were collected through the sampling port. The septum was removed and a sterile 10 mL serological pipette was inserted to the bottom of the container. As suction was applied, the pipette was lifted up in a spiral motion to sample the medium throughout the container.

Resident organisms came from hydroponic wheat rhizosphere of an ongoing EGC experiment at Advanced Life Sciences Support, Hangar L, Kennedy Space Center. A section of root mat was cut with scissors and interstitial medium was squeezed by hand into a beaker: this extract was the resident inoculum. Ten mL of mixed resident inoculum was inoculated into appropriate experimental containers immediately before seedling placement, on 4 June 1996 (Day -7 of the experiment). Organisms in remaining resident inoculum were identified and scored on a 1-5 relative abundance scale (1=rare, 5= very abundant). Plants and resident organisms were then left undisturbed for one week before invader inoculation. Hydroponic medium samples were collected on 11 June 1996 (Day 0), prior to invader inoculation, and examined as above.

Invader inoculum sources were 3 ponds in the Merritt Island National Wildlife Refuge, adjacent to KSC: ponds were selected to encompass a salinity range, and were designated as fresh (<1 ppt), brackish (7 ppt), and salt (16ppt). We expected that fresh, brackish and saltwater inocula would provide a range of invasion intensity, given that organisms adapted to saline conditions would not readily adapt to the hydroponic medium (< 1 ppt).

Invader inocula were collected on 10 June 1996 by simply filling a wide-mouth 1 L jar with submerged aquatic vegetation and pond water. Samples were placed on ice until return to the laboratory, where they were held at room temperature overnight. The next morning (Day 0), samples were shaken vigorously and sieved on 0.5 mm mesh to separate plants and macroorganisms from inocula. This procedure was repeated with approximately 50 mL of the sieved water to further extract organisms from plants. Extract was then mixed and 10 mL were pipetted into appropriate jugs. Organisms in invader inoculum were identified and scored for relative abundance as above.

Combination of the above treatments resulted in the following treatment combinations: freshwater invaders, non-resident (FNR); freshwater invaders, resident (FR); brackish invaders, non-resident (BNR); brackish invaders, resident (BR); saltwater invaders, non-resident (SNR); saltwater invaders, resident (SR).

Further samples were collected from hydroponic medium and examined microscopically (as above), using 10 mL pipettes (as described above), on the following dates: 14 June (Day 3), 25 June (Day 14), and 8 July (Day 27). The experiment ended 9 July (Day 28), when plants were removed after 28 days of hydroponic growth. Plant roots were cut with scissors and placed in a sterile, 50-mL centrifuge tube containing 30 mL sterile saline solution (0.85% NaCl). Sterile glass beads were added and samples were inverted 10 times to extract invertebrates from rhizosphere. A 10-mL subsample was taken for microscopic examination, as above. Plant roots and shoots were dried at 70° C for 48 hrs and weighed.

Data were analyzed by both univariate and multivariate techniques. Invertebrate species number and plant dry weights were analyzed by ANOVA and Tukey's HSD (SAS 1990). Invertebrate relative densities (Day 27 media and rhizosphere samples) were analyzed by detrended correspondence analysis (DCA) and cluster analysis (using centroid distances) was run for rhizosphere results.

1.3 RESULTS

Wheat plants grew to approximately 2 g total dry weight. Some plants that received resident inocula had brown roots and lesser root mass, probably related fungal infection, but root, shoot, and total dry weights were not significantly different among treatments or blocks.

Organisms present in resident, freshwater, brackish and saltwater inocula are listed in Table 1. Note that all resident organisms had been observed in previous samples from CELSS rhizospheres. In addition, two other rotifers, *Lecane inermis* and *Lecane hamata*, have been observed in previous CELSS rhizosphere samples and were observed in subsequent samples from jugs inoculated with residents: it is likely that they were present as resting eggs or were very rare in initial inoculum.

Composition in hydroponic media (and presumably rhizosphere) underwent successional change. For example, *Actinophrys sol* was numerous in initial resident inoculum, but faded away during the experiment, likely related to static conditions in the experimental jugs compared to flow-through conditions in EGC and BPC experiments.

Species number in hydroponic media decreased after inoculation in all experimental treatments (Figure 1). Resident inoculum contained 8 species at Day -7, but media inoculated with residents had an average of 3 species at Day 0, immediately prior to addition of invaders. Fresh and saltwater species numbers dropped most sharply after inoculation (Figure 1). Jugs that were not inoculated with resident organisms at Day -7 were uncontaminated at Day 0.

Species numbers generally increased slightly following Day 0: all jugs had received invaders, and ½ of the jugs had also received residents one week earlier. As expected, saltwater and brackish invaders contributed little to species number (Figure 1). The greatest increase occurred in FNR media, reaching an average of 15 species at Day 14. However, FR and FNR treatments had similar species numbers by Day 27 (Figure 1).

Invader source had a significant effect on species number in hydroponic medium (ANOVA; $p=0.0001$), and jugs receiving freshwater invaders had significantly more species than jugs receiving brackish or saltwater invaders ($p=0.05$, Tukey's HSD). The presence/absence of resident organisms prior to invasion had no significant effect on species number. Blocks were also not significantly different, and no significant interaction existed between the two main treatments.

Rhizosphere samples were analyzed separately from media samples. Invader source, resident presence/absence, and source-resident interaction significantly affected species number (ANOVA; $p=0.0001$, 0.005, 0.014, respectively). Communities invaded by freshwater organisms had more species than those invaded by saltwater and brackish organisms, and communities with resident organisms had more species than those without residents (Tukey's HSD, $p=0.05$). Also, rhizosphere communities were significantly more diverse in FR jugs than other jugs (Figure 1).

Multivariate analyses also indicated differences among treatments. Detrended correspondence analysis (DCA) of rhizosphere data yielded three groupings along the first axis: BNR and SNR communities, BR and SR communities, and FR and FNR communities (Figure 2). The DCA results were consistent with cluster analysis of rhizosphere data (Figure 3). Finally, the same general pattern among treatments was shown by DCA of Day 27 media + rhizosphere data, although media and rhizosphere patterns were separated, especially for freshwater treatments (Figure 4). This analysis indicated that treatment effects were consistent in different micro-habitats, and that media samples yielded different composition than rhizosphere samples, as was expected.

Inspection of FR and FNR rhizosphere community composition revealed that FR and FNR treatments shared 12 of the 24 species observed in both treatments (Table 1). Of those 12 species, 6 were in resident inoculum or have been observed in resident communities previously (Table 1). This overlap in invader and resident composition is consistent with previous chance colonization of BPC and EGC rhizospheres from regional freshwater sources. Nine other species occurred in FR rhizospheres only (although infrequently), and 3 of those 9 species (*Cephalodella gracilis, Cyclidium, and Stylonichia*) were in the initial resident inoculum. The other six species have never been observed in samples of CELSS rhizosphere and probably entered as freshwater invaders. Three genera were exclusively observed in FNR rhizospheres: *Litonotus, Hartmanella, and Onychodromus*, indicating that they originated from invader inocula.

Fresh, brackish, and saltwater invaders were used because we anticipated a gradient in invasion strength due to varying tolerance of low-salinity culture medium. Invader sources did vary in invasion strength, but not due to osmotic stress. The fractions of fresh, brackish, and saltwater species that survived to Days 27 or 28 were comparable (52%, 58%, and 56%, respectively). Freshwater invader inoculum was more diverse than brackish or saltwater inocula, thus providing greater invasion pressure, regardless of osmotic tolerance among species from different habitats.

Species were individually judged for their ability to colonize the experimental systems. The compatibility of the environment and the effect of biotic interactions were inferred from data for rhizosphere samples (Day 28) or media samples at Day 27 (Table 2). We considered presence/absence at 27 or 28 days post-invasion to be a reasonable test of success for communities composed of protists and microscopic metazoans. We judged taxon presence in 3 or 4 replicate jugs of a treatment to indicate success: presence in 1 or 2 replicates was considered unclear evidence of success or exclusion.

One-half (51%) of the invader species clearly survived 28 days: more invader species were present at Days 27 and 28 than residents (Table 2). We considered this evidence of successful colonization for those species. In addition, consider the number of species present in FNR treatments (Figure 1): clearly, invaded jugs accrued invading species. However, about one-third (39%) of all invader species did not successfully invade the hydroponic habitat (by our criterion of presence at Days 27 or 28). Environmental conditions apparently excluded those species, assuming that biotic interactions among members of the inoculated community did not alter their survivorship.

About two-thirds of the invaders (68%) successfully colonized with no apparent effects by residents, and 3 species (*Blepharisma, Colurella*, and *Encentrum*: 8%) were more successful in the presence of residents. Only 5 (13%) were clearly excluded from habitats containing residents, and four other species (11%) may have been excluded: mechanisms of exclusion are not known. Most invaders (76%) could successfully colonize systems with residents, and the number of species excluded by resident presence were outnumbered by those excluded by environmental conditions.

1.4 DISCUSSION

Can invaders successfully colonize hydroponic systems that lack resident organisms? The majority of species successfully colonized non-resident rhizospheres, although about 1/3 of the species did not. Environmental conditions apparently excluded some species, assuming that biotic interactions among members of the inoculated community did not alter their survivorship. Therefore, environmental conditions partially limited invertebrate community composition, and this effect was not limited to species

from brackish or saltwater habitats: 9 of 24 excluded species were in initial freshwater inoculum.

Given the partial overlap in invader and resident taxonomic composition, it is likely that regional habitats served as source pools for some species when they first colonized the facility and became current rhizosphere residents. It is also likely that similar colonization events will continue, albeit at a low rate due to isolation of hydroponic habitats within the facility. This process is suggested by the observation of *Floscularia* and *Stentor* in initial resident inoculum: neither genus had been observed previously in CELSS hydroponic systems and are probably recent colonists. Other organisms were successful upon experimental invasion but have not been observed in resident communities, probably because they disperse less readily.

Can invaders successfully colonize hydroponic systems that contain residents? About 3/4 of the invaders did, and environmental conditions excluded more species than biotic interactions. Lodge (1993) pointed out that reliable statistics on invasion success rates are rare for a variety of reasons, including difficulties in recording unsuccessful invaders, biases to easily-observed species, paucity of data on many taxa, and a focus on potentially-successful species in planned invasions for biological control. Nevertheless, Lodge (1993) cited values of 10% (Williamson 1989) and 1-24%, ranging up to 46% for one taxon (Vermeij 1991). The much higher invasion rate (76%) attained in our experiment supports our conclusion that rhizosphere communities were invasible.

Resident communities were relatively simple, potentially containing up to 12 species. Simple communities should be more invasible than speciose communities, related to cumulative effects of resident predators and competitors on invaders (Lodge 1993). This logic most clearly applies to single invaders entering speciose resident communities. In the case of a speciose invasion of a simple resident community, cumulative effects of invader predators and competitors might be expected to exert strong pressure on residents. However, resident species were not excluded and did not clearly have reduced densities upon invasion. We find it difficult to attribute the invasibility of the communities to biotic interactions by diverse invaders.

The greater diversity of FR treatments (versus FNR), and the similarity of FR and FNR in multivariate analyses and species lists all indicate that invasion did not simply displace resident communities. Likewise, resident communities did not seem to exclude more than a few invader species (e.g., *Litonotus* and *Hartmanella*). Therefore, it seems unlikely that biotic interactions were strong enough to yield detectable changes in community composition. If anything, invaders supplemented the resident community, and may have reinforced resident populations in cases of taxonomic overlap (e.g., *L. hamata, L. inermis, R. rotaria*). Lodge (1993) stated that "many, if not most, colonists have little impact on invaded communities," and Vermeij (1991) showed from paleobiological evidence that invasion often simply increases species richness. Of course, strong direct and indirect interactions have been documented, with predation and habitat change most often invoked as mechanisms of interaction between invaders and residents; competition is more difficult to demonstrate and rarely invoked (Lodge 1993). Predators and potential competitors existed in both invader and resident communities, but neither interaction strongly affected community composition. It is unlikely that invertebrates would have significantly changed rhizosphere habitat.

Crawley (1987) stated that "a community is invasible when an introduced species is able to increase when rare," and that "it is impossible to to recognize the existence of a vacant niche without the empirical attempt to establish a given species in a given environment." By these criteria, our results show that rhizosphere communities were invasible many times over, because multiple open niches exist. Hydroponic rhizospheres appear to contain vacant niches, in that invader organisms successfully colonized without displacing resident organisms. An invasible community is often considered to have an open niche for the invader, although it is usually difficult to establish that open niches exist. Crawley (1987) listed three problems with the concept of open niche: (1) investigators measure niche differently (i.e., niche is in the eye of the beholder); (2) invasion alone does not indicate an open niche, because invaders may displace residents, rather than fill an open niche; and (3) apparently unexploited resources (perceived open niches) may not be exploitable for hidden reasons (e.g., low resource quality, etc.). We avoided these three pitfalls by making no assumptions about niche dimensions, tracking both resident and invader

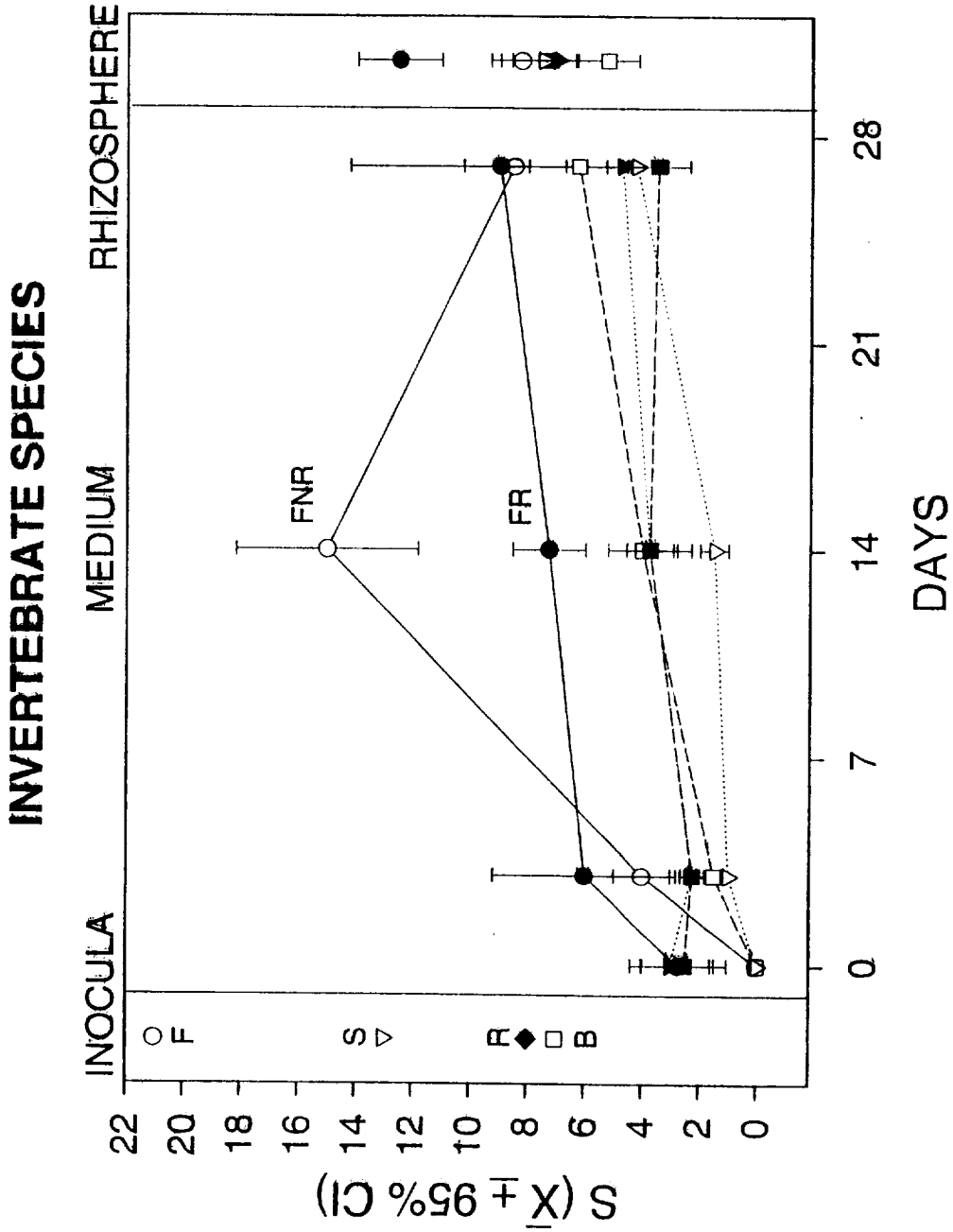
populations, and by evaluating invader success rather than perceived resources.

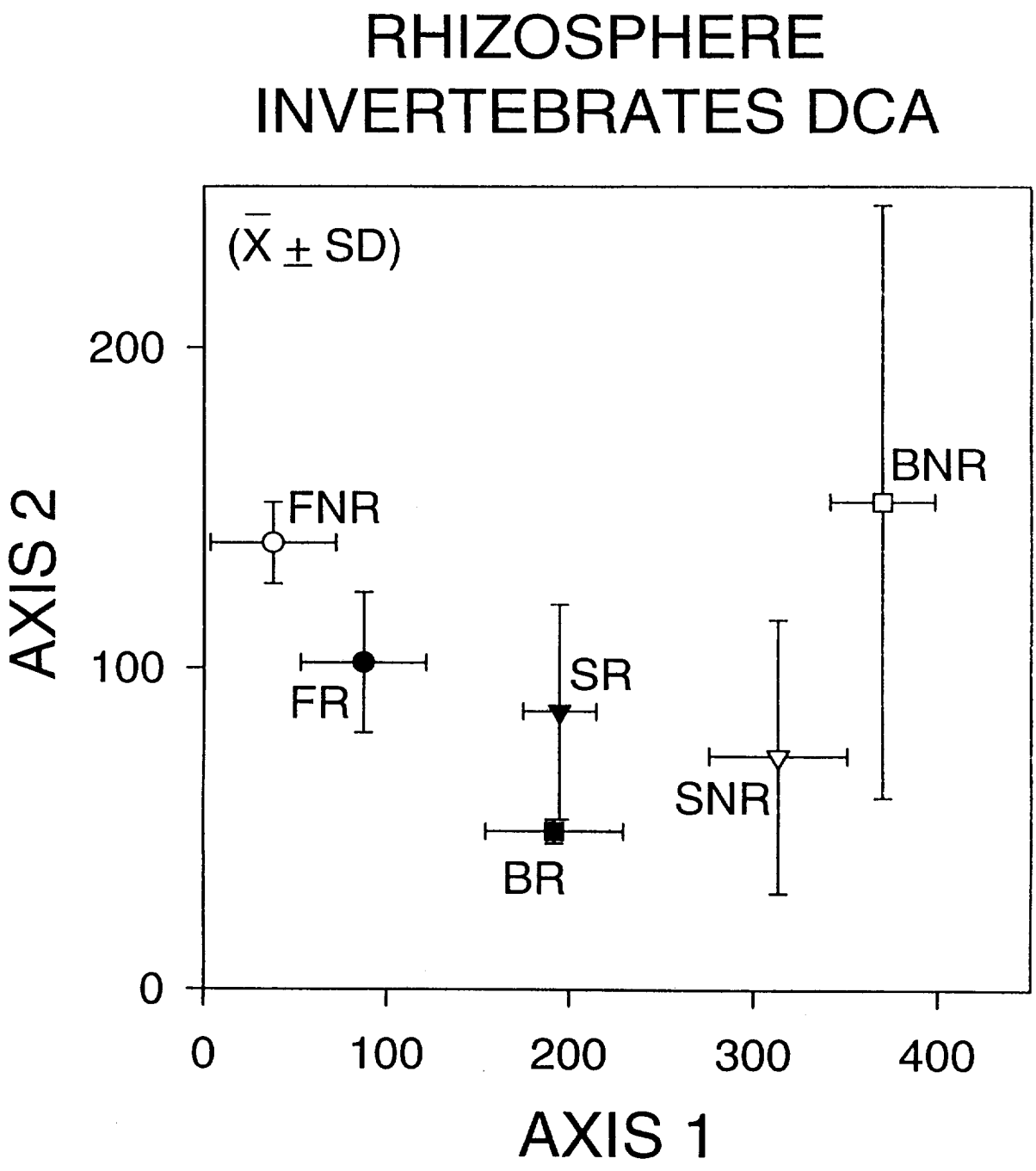
An alternative to multiple open niches may explain the invasibility of the rhizosphere communities: abundant resources. Bacterial densities in the CELSS rhizospheres are commonly about 10^{10} cells / cm^2 root surface area (Strayer 1994). Some residents may occupy niches that largely overlap or coincide with some invader niches, but competitive exclusion would not take effect until resources became limiting; an unlikely event at such high bacterial density. Limiting resources would not occur until either bacterivory became intense (very high invertebrate density), or microbial production was reduced (low nutrient levels, etc.). Experiments with more diverse and dense resident communities and varying resource availability may be needed to detect competitive exclusion.

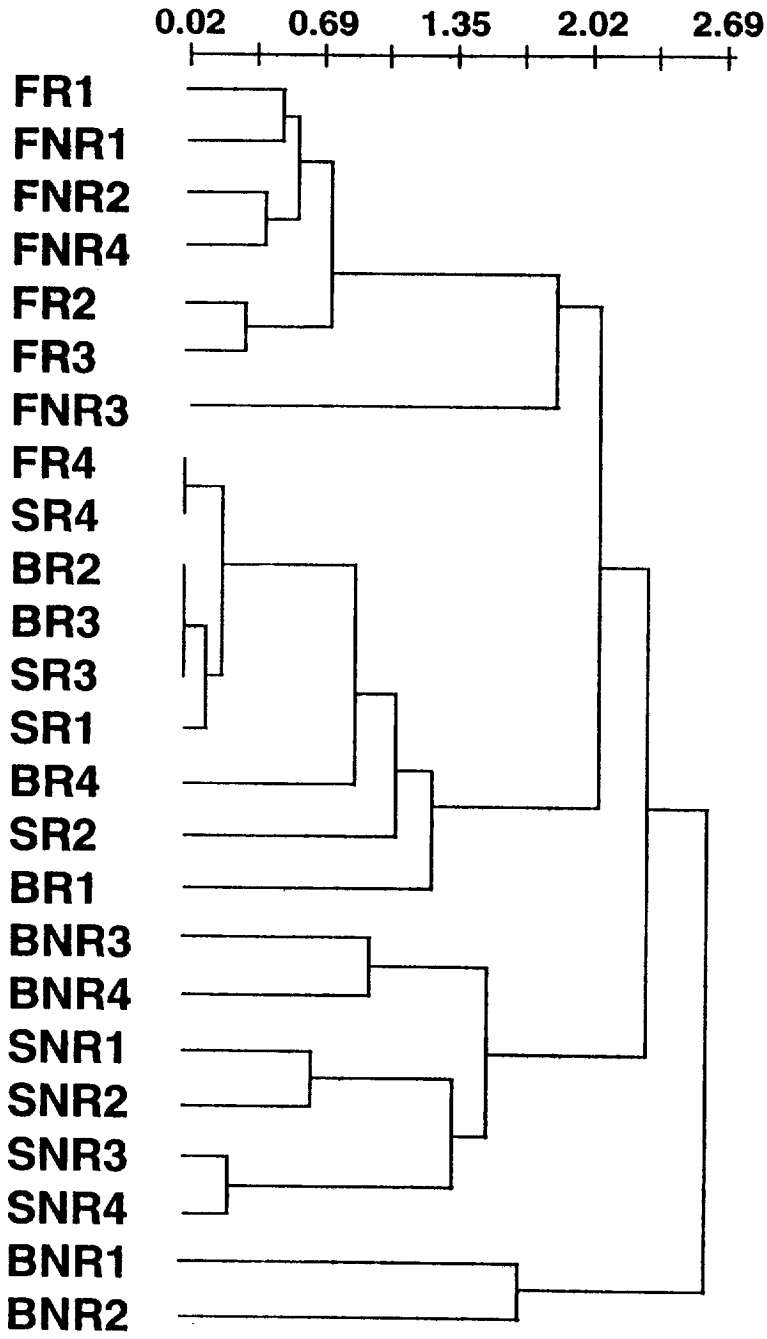
Invaded communities attained different end states. Our experimental design focused on the invasibility of rhizospheres with and without the presence of resident organisms: we can not compare invaded end states to uninvaded end states to statistically evaluate differences. Likewise, we cannot evaluate temporal patterns as a measure of stability, because media and rhizosphere communities differed and rhizospheres were sampled only at the end. However, the rhizosphere environment (without residents) was invasible by about one-half of the tested organisms, and invasion of those environments by organisms from different sources led to different outcomes. This result is not unexpected, given different assemblages in original inoculum.

More importantly, invasion by different communities led to different outcomes despite the presence of residents. Differences among invader source treatments were lessened by the common denominator of residents, but remained. The similarity of freshwater-invader communities in multivariate analyses indicates that invaders were important in characterizing both treatments: this pattern would not exist if residents had resisted invasion. Brackish and saltwater treatments with residents were more clearly separated from those without residents: residents played a greater role in defining those communities, due to the lesser numbers of invader species in brackish and saltwater inocula.

1.5 LIST OF FIGURES


INVERTEBRATE SPECIES
INOCULA
MEDIUM
RHIZOSPHERE
FNR
FR
F
S
R
B
S (X̄ ± 95% CI)
DAYS
0
2
4
6
8
10
12
14
16
18
20
22
0
7
14
21
28


RHIZOSPHERE
INVERTEBRATES DCA
(X̄ ± SD)
AXIS 2
AXIS 1
0
100
200
0
100
200
300
400
FNR
FR
SR
BR
SNR
BNR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FNR1
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FNR4
FR2
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BR2
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BNR1
BNR2

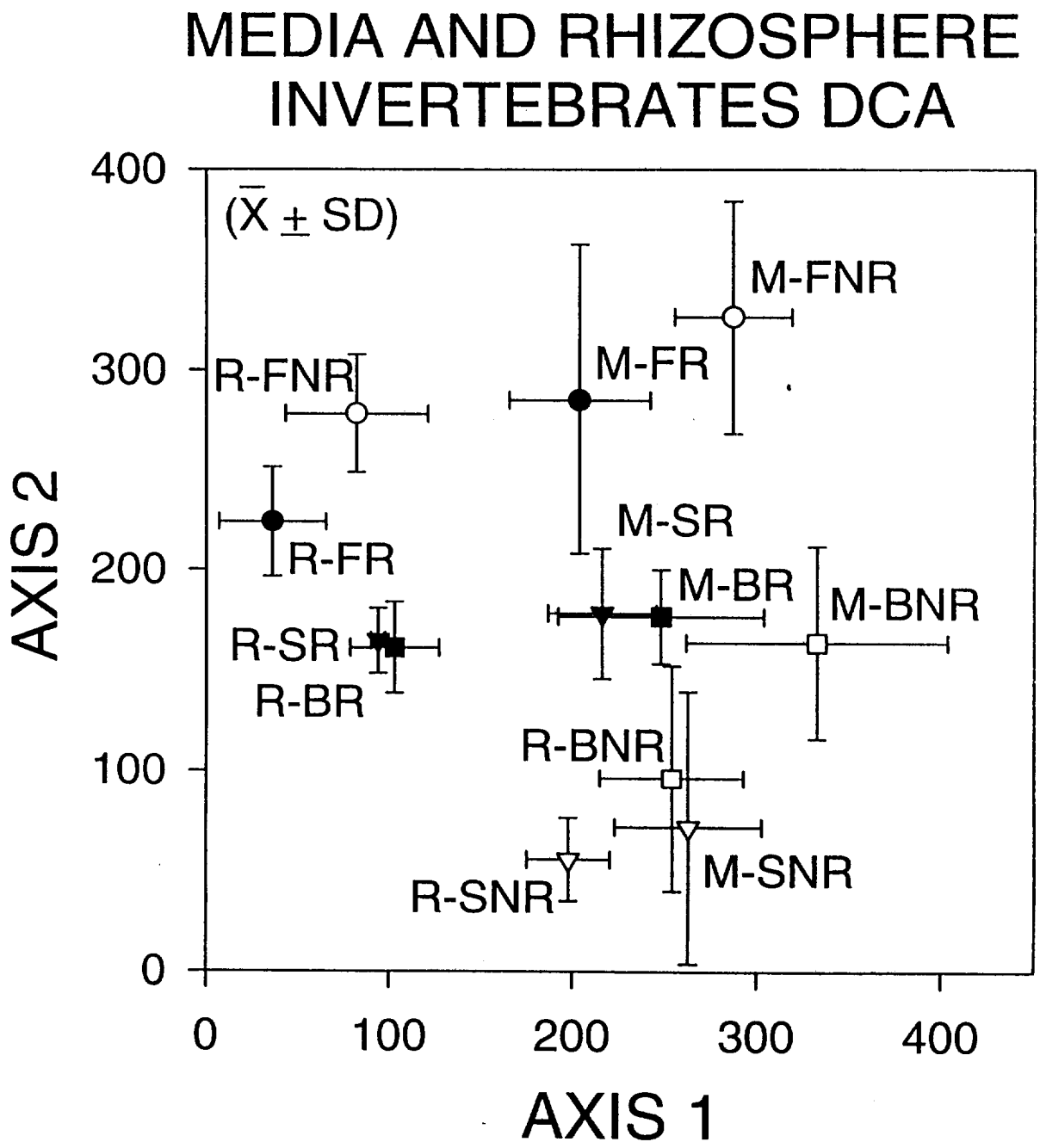
MEDIA AND RHIZOSPHERE
INVERTEBRATES DCA
(X̄ ± SD)
M-FNR
M-FR
R-FNR
M-SR
R-FR
M-BR
M-BNR
R-SR
R-BR
R-BNR
M-SNR
R-SNR
AXIS 2
AXIS 1
0
100
200
300
400

Table 1. Invertebrate taxa observed in initial resident inoculum, freshwater, brackish, and saltwater invader inocula, and rhizosphere samples after 28 days. An X indicates presence; multiple X's for rhizospheres indicate the number replicate jugs in which taxa was observed (maximum = 4).

					RHIZOSPHERE (DAY 28)					
TAXA	RESIDENTS	FRESH	BRACKISH	SALT	FR	FNR	BR	BNR	SR	SNR
Actinophrys	X							X		
Arcella					XXXX	XXX				
Aspidisca					X					
Blepharisma					X		XXX		XX	XX
Bodo saltans	X				XX	X	XX	XX	XX	XXXX
Bodo sp.	X									
Bryophyra		X								
Cephalodella	X	X			XX		XX		XXX	X
Chaetonotus		X			X					
Cinetochilum								XX		X
Coleps		X		X						
Colurella		X	X		XXXX	XXXX			XX	
Cyclidium	X				XX		XXXX	X	XXX	XX
Dileptus anser								X		
Encentrum				X			XXX		XX	X
Eosphora		X								
Euchlanis dilatata		X								
Euglena		X								
Euplotes					X	XX		X		
Floscularia	X			X						
Gonostomum					X			X		XX
Hartmanella						XXX				
large ciliate			X							
Lecane asthena								XX	XX	
Lecane cornuta					X					
Lecane hamata					XXXX	XXX	X		X	
Lecane inermis		X	X		XXXX	X	XXX		XXXX	X
Lecane obtusa							X			XXX
Lecane pyriformis					XXXX	XXX				
Lecane tethis										XXX
Lepadella					XX	X				XX
Limnias		X								
Litonotus				X		XXXX		X		
Mayorella		X			XXX	XXX	XX		X	
Microdalyiella			X	X						
nematode		X		X	XXXX	X	X			
Notomatta		X			XX					
Onychodromus						X				
ostracod		X		X						
Peranema								XXX		XXX
Pleuronema	X									
Rotaria rotaria		X			XXXX	XX	XXXX		XXXX	
small ciliate		X	X	X	XX	X		XX		XXX
snail			X							
Squalophyra		X								
Stenostomum				X						
Stentor	X	X								
Stylonichia					X					
Trachelophyllum		X								
Trichocerca		X								
Vaginocola								X		X
Vorticella		X	X				XXX	XXX	XX	X

1: FR = freshwater invaders, residents present; FNR = freshwater invaders, no residents present; BR = brackish wa invaders, residents present; BNR = brackish water invaders, no residents present; SR = saltwater invaders, resid present; SNR = saltwater invaders, no residents present.

2: In addition to taxa listed above, others were observed in media samples at Days 3, 14, or 27, including: *Actinosphaerium, Chilodonella, Colpidium, Cyphoderia, Difflugia, Dissotrocha, Gastrostyla, Gromia fluvialis, Halteria, Heteromonas, Mesodinium, Metopus, naidid oligochaete, Nuclearia, Oxnerella maritima, Pamphagus, Salpingoeca, Tetrahymena, Thecamoeba, Urocentrum turbo, Uroleptis.*

Table 2. Summary of invasion patterns for the rhizosphere environment and biotic interactions between invaders and resident communities. The rhizosphere environment was judged to be compatible for invaders if they survived in non-resident treatments after 27 or 28 days. Biotic interactions were judged for those invaders that survived in non-resident treatments.

Environmental Compatibility for Invaders

	Number	Percent of Invaders
Survived:	31	51%
Not Survived:	22	36%
Unclear (rare):	8	13%
Total Invaders:	61	100%
Residents and Inferred Residents:	12	

Biotic Interactions for Invaders that Survived Environmental Conditions

	Number	Percent of Invaders
Excluded by Residents:	5	16%
Possibly Excluded by Residents:	4	13%
Promoted by Residents:	3	10%
Unclear (mixed or rare):	19	61%
Total:	31	100%

LYSOZYME ANALYSES (GONZALEZ et al. 1993, VRBA et al. 1993) ARE NEITHER PROTISTAN- OR BACTERIVORE-SPECIFIC.

2.1 INTRODUCTION

Microbial food webs have been acknowledged for some time as being important in aquatic ecosystem energetics and materials cycling (Azam et al. 1983, Cole et al. 1988, Pomeroy and Wiebe 1988). Protozoans are most often considered the major grazers of bacteria in aquatic systems, but metazoans also feed on bacteria and can indirectly regulate bacterial composition and productivity via predation on protozoans (Porter et al. 1985, Berninger et al. 1991, Jurgens et al. 1994). Bacterivory by protozoans and metazoans has most often been estimated by quantifying fluorescently- or radio-labelled bacteria, or by inference from changes in bacterial density (e.g., Sherr et al. 1987, Nygaard and Hessen 1990, Jurgens et al. 1994). Various problems exist with these techniques, and alternative methods that are based on analysis of lysozymes have been described (Gonzalez et al. 1993, Vrba et al. 1993). We use the term "lysozymes" here to include all forms of the enzyme that hydrolyzes peptidoglycan, regardless of specific nomenclature (Stryer 1981, Cabezas 1989).

Gonzalez et al. (1993) developed the acid lysozyme (L_{ACID}) assay to measure lysozyme activity in protistan cell lysates at acid pH, which has been demonstrated to occur in protistan food vacuoles (Sleigh 1989). Gonzalez et al. (1993) demonstrated maximal L_{ACID} activity at pH 4.5, and a strong relationship between bacterivory rates (fluorescently-labelled bacteria intake) and acid lysozyme activity in a variety of marine water samples and protistan cultures.

While clearly a valuable tool for measuring bacterivory by some protistan isolates, the L_{ACID} assay must pass another test for it to be considered protistan-specific: metazoan bacterivores must not produce L_{ACID} as well. Gonzalez et al. (1993) argued that the L_{ACID} assay is protistan specific because marine bacterial exoenzymes are inactive at pH < 5 and function maximally at seawater pH. Water samples often contain a variety of metazoan taxa, especially samples from inland, estuarine, or benthic marine waters. Some metazoan invertebrates (e.g., rotifers) are comparable in size to some protists, and may not be easily separated from protists in samples prior to L_{ACID} analyses. If metazoan bacterivores contribute to measured "protistan" L_{ACID} activity, protistan bacterivory would be overestimated and metazoan importance underestimated.

In addition, protistan L_{ACID} must be shown to differ from protistan and metazoan lysozymes that can act at acid pH but that may be present for reasons other than bacterivory. Lysozymes hydrolyze the β(1-4) glycosidic bond between N-acetylmuramic acid (NAM) and N-acetylglucosamine (NAG) in peptidoglycan, but also hydrolyze chitin, which is composed of only NAG molecules joined by β(1-4) glycosidic bonds (Stryer 1981). Therefore, protistan and metazoan predators of chitin-bearing prey (e.g., rotifers, nematodes) and chitin-bearing metazoans that molt should have lysozymes that hydrolyze the analog substrate, 4-methylumbelliferyl β-D-N,N',N''-triacetylchitotriose (MUF-CHT), used by Gonzalez et al. (1993). Chitotriose is a trimer of NAG molecules and an intermediate breakdown product of chitin digestion (Vrba et al. 1993, Gooday 1990). If organisms have lysozymes that are not involved in bacterivory but that react with MUF-CHT, the method of Gonzalez et al. (1993) would overestimate protistan bacterivory.

Overlap in metazoan and protistan sizes and the presence of chitinolytic lysozymes should also affect the method of Vrba et al. (1993), which uses 4-methylumbelliferyl β-N-acetylglucosaminide (MUF-NAG) hydrolysis as a measure of protozoan bacterivory. The substrates used by Gonzalez et al. (1993) and Vrba et al. (1993) differ only in the presence of two additional NAG monomers: otherwise the MUF-CHT and MUF-NAG substrates are identical (Sigma Chemical Co. Product Structure Data Sheets M5639 and M2133). The enzyme assayed by Vrba et al. (1993), β-N-acetylglucosaminidase, is a lysozyme by definition (Stryer 1981). We did not specifically test the method of Vrba et al. (1993), but the fact that both methods assay lysozymal cleavage of the same bond suggests that our findings for the method of Gonzalez et al. (1993) apply to the method of Vrba et al. (1993) as well.

We began this study with the intent of testing the protistan-specificity of L_{ACID} analysis. We wished to know if the method would discriminate protistan from metazoan bacterivory in complex communities. Alternatively, we thought that a general measure of community bacterivory might still prove useful. In the process of explaining our results, we more closely examined the chemistry underlying the Gonzalez et al. (1993) and Vrba et al. (1993) methods.

2.2 MATERIALS AND METHODS

Organisms were obtained from three small ponds on the Merritt Island National Wildlife Refuge, Florida and from Carolina Biological Supply Co. The ponds varied in salinity (<1, 7, and 16 ppt). A pond sample was collected by scooping or pushing submerged aquatic vegetation into a 1 L plastic bottle. The bottle was filled with pond water and placed on ice until return to the laboratory for processing. A second bottle was also filled with pond water and chilled to ensure sufficient water for maintaining animals in the lab.

We expected *a priori* that several genera would not have L_{ACID}, based on basic information about feeding: *Stentor* is predacious on other protists (Pratt and Cairns 1985); *Chilomonas* (a cryptomonad) is not phagotrophic (Lee et al. 1985); tardigrades have stylet mouthparts and feed by piercing plants or small metazoans (Pennak 1989); and cyclopoid copepods are raptorial feeders (Pennak 1989).

Samples were held at room temperature during processing. Each field sample jar was vigorously shaken to dislodge organisms attached to plants. Aliquots of field-collected and cultured samples were then examined in a Petri dish with a dissecting scope. Animals were individually isolated by micropipetting them in a series of filter-sterilized (0.2 μm) pond or culture water. Three to four sequential transfers were used to obtain an isolate for acid-lysozyme analysis.

Isolated organisms were sonicated and analyzed according to Gonzalez et al. (1993). We measured fluorescence at 360 nm with a Perkin-Elmer LS 50 B luminescence spectrometer (excitation at 330-400 nm, emission filter = 470 nm. Readings from boiled controls (1 per extract) were subtracted from results to correct for background fluorescence. Results were expressed as μM MUF / mL extract. Expression as bacteria mL^{-1} h^{-1} would require calibration with ingested fluorescently-labelled bacteria (Gonzalez et al. 1993); an unnecessary step in a simple test of the presence of acid lysozyme among taxa. In addition, we did not standardize extracted biomass among taxa, which would be expected to affect magnitude of results. We focused at this point on the presence or absence of the enzyme in diverse taxa, not the magnitude of results.

2.3 RESULTS

Results are listed in Table 1: taxonomy is per Pennak (1989). Values with confidence intervals overlapping a value of 0 (no activity) were considered to indicate a negative result. Genera listed twice were analyzed on separate occasions.

Of the 6 protistan genera tested, only *Stentor* unequivocally lacked L_{ACID} (Table 1): a response we predicted because *Stentor* is a predator of other protists. Results for *Actinosphaerium* were low, and in one case variable enough to be considered a negative result by our criterion. The latter is probably a result of low density of isolated organisms (26 individuals isolated in 6 mL) in the analyzed extract. Two values are listed for *Paramecium*: the first for organisms isolated in original culture solution, and the second from organisms isolated in an optically-clear culture solution. Because we expected *Paramecium* to have L_{ACID}, we were surprised to have a negative result for organisms in the original culture solution. Analysis of organisms in clear solution indicated that *Paramecium* do in fact have L_{ACID}, and that dissolved organic compounds (e.g., humic and tannic acids) apparently interfered with fluorescence detection at 360 nm.

We analyzed 10 micrometazoan taxa (Platyhelminthes, Rotifera, Nematoda, and Tardigrada). Nine had L_{ACID}, including organisms we thought would not have L_{ACID} (tardigrades and cyclopoid copepods). *Brachionus* was marginally positive, probably due to low density of extracted individuals. Another rotifer,

Lepadella, had a greater mean value than *Brachionus*, but subsamples varied in activity enough to be considered negative. *Stenostomum* was also low in density, which may explain the mixed results among extracts. One unidentified nematode extract was marginal, while the other sample was clearly positive, again probably related to density effects on detection limit.

All 12 of the macrometazoan genera (Annelida and Crustacea) were clearly positive (Table 1). Greater biomass for some of these genera was probably reponsible for greater mean values. Surprisingly, cyclopoid copepods clearly had L_{ACID}.

In summary, 5 of 6 protistan genera tested had L_{ACID}: the one lacking L_{ACID} (*Stentor*) was predicted. However, one other protistan genus, *Chilomonas*, was predicted to lack L_{ACID} but had it. Of the 21 metazoan taxa tested, 20 (95%) had L_{ACID}. The one genus (*Lepadella*) with activities not significantly different from 0 may actually have L_{ACID}, but biomass may have been too low to get a clear reading.

2.4 DISCUSSION

Acid lysozyme analysis is not protistan-specific. This conclusion stands even if we discount the genera that had marginal or low activities. All five of the metazoan phyla tested were positive for L_{ACID}. Clearly, L_{ACID} analysis of field samples can overestimate protistan bacterivory if metazoans are in the extracted sample, especially if whole water samples (e.g., Gonzalez et al. 1993) or large-mesh plankton netting are used. Likens and Gilbert (1970) found that 35 μm mesh is needed to quantitatively sample rotifers (i.e., remove rotifers from filtrate). Use of mesh > 35 μm to exclude metazoans (e.g., Vrba et al. 1996) will permit small metazoans to pass into samples, potentially leading to overestimates of protistan bacterivory. Although it may exclude some large protists, use of 20-μm (e.g., Sherr et al. 1992) or 35-μm mesh will more clearly isolate bacterivorous protists from metazoans.

Acid lysozyme analysis is not bacterivore-specific. Gonzalez et al. (1993) correctly stated that peptidoglycan only occurs in eubacteria cell walls, but lysozymes hydrolyze β(1-4) glycosidic bonds of both peptidoglycan and chitin (Stryer 1981). Any samples that contain organisms with chitinolytic activity will overestimate "protistan" bacterivory when analyzed for L_{ACID} by the method of Gonzalez et al. (1993). In addition, it is possible that some protistan predators use lysozymes to degrade chitin of captured prey (e.g., rotifers, nematodes, gastrotrichs, etc.) and peptidoglycan of bacterial cell walls. Thus, L_{ACID} cannot be expected to discern between protistan and metazoan bacterivory, let alone bacterivory and chitinolysis in general.

Although we did not test the method of Vrba et al. (1993), we must infer the same conclusion for their method. The methods of Gonzalez et al. (1993) and Vrba et al. (1993) both analyze enzymatic hydrolysis of the β(1-4) glycosidic bond attaching MUF to NAG: Gonzalez et al. (1993) uses NAG trimers (chitotriose), while Vrba et al. (1993) uses NAG monomers. The methods differ (e.g., sonication of cells versus whole-cell analysis for extracellular enzyme activity), but the basic lysozyme-glycosidic bond reaction is central to both methods. Vrba et al. (1993) considered interference by bacterial chitinolytic enzymes (lysozyme), but did not rule out invertebrates as sources of chitinolytic lysozyme. Given that chitin-bearing invertebrates that molt may release lysozyme into the water, the method of Vrba et al. (1993) may be subject to the dual problems of non-specificity for Protista and bacterivory. In addition, it is possible that some protistan predators use lysozymes to digest chitin-bearing prey, which would further overestimate protistan bacterivory by lysozyme analysis.

The fact that "acid lysozyme" has maximal activity at pH 4.5 is not by itself indicative of a protistan-specific assay. Multiple metazoan phyla exhibited lysozyme activity at that pH in our analysis. Gonzalez et al. (1993) did not compare protistan and invertebrate metazoan lysozyme pH optima, but only compared protistan and bacterial lysozyme pH ranges.

Our results indicate two additional problems with the Gonzalez et al. (1993) method. First, the color interference with *Paramecium* culture solution indicates that natural waters colored by dissolved organic matter may be difficult to analyze by this method. Secondly, *Chilomonas* had L_{ACID}, although *Chilomonas* is a cryptomonad that does not feed phagotrophically (Lee et al. 1985). This result is consistent with

extracellular release of lysozyme, as measured by Vrba et al. (1993). Is it possible that intracellular L_{ACID} and extracellular β-N-acetylglucosaminidase are two versions of the same lysozyme, operating at different pH optima? Vrba et al. (1996) observed both high- and activity enzyme kinetics, and offered extracellular release of vacuolar enzymes as a possible explanation for low-activity enzymes.

Enzymatic assays of bacterivory offer the potential to mitigate errors introduced by other methods (Gonzalez et al. 1993), but only if enzymatic assays themselves are less likely to introduce greater error. Our results indicate that protistan bacterivory could be grossly overestimated by lysozyme analyses (e.g., Gonzalez et al. 1993, Vrba et al. 1993) of samples more complex than protistan isolates. In addition, potential chitinolysis by predatory protistans may introduce error in protistan isolates. At this point, the tradeoffs involved in deciding on a method to analyze bacterivory in aquatic habitats do not point to abandonment of other approaches in favor of lysozyme analyses.

Table 1. Acid lysozyme analysis results among invertebrate taxa. Results include organisms isolated from both field and cultured samples. Each mean represents two analyzed extracts from one sample, with 95% confidence intervals (CI). See text for further descriptions.

	uM MUF / mL	
	Mean	95% CI
Phylum Sarcomastigophora		
Subphylum Sarcodina, Class Actinopoda		
Actinosphaerium	0.063	0.080
Actinosphaerium	0.051	0.005
Subphylum Sarcodina, Class Rhizopoda		
Amoeba proteus	0.313	0.007
Amoeba proteus	0.262	0.148
Subphylum Mastigophora, Class Phytomastigophorea		
Peranema	0.493	0.002
Chilomonas	1.974	0.227
Phylum Ciliophora		
Paramecium (brown soln)	-0.037	0.049
Paramecium (clear soln)	8.509	0.355
Stentor	-0.005	0.002
Stentor	0.001	0.001
Phylum Platyhelminthes, Class Turbellaria		
Microdalyiella	0.299	0.031
Stenostomum	0.010	0.003
Stenostomum	-0.018	0.007
Phylum Rotifera		
Brachionus	0.007	0.005
Lecane	0.020	0.002
Lepadella	0.024	0.030
Philodina	0.783	0.082
Rotaria	0.018	0.007
Phylum Nematoda		
Cephalobus	2.489	1.495
Unidentified nematode	0.008	0.005
Unidentified nematode	0.027	0.002
Phylum Tardigrada		
Unidentified tardigrade	0.472	0.252
Phylum Annelida, Class Oligochaeta		
Stylaria	17.588	0.074
Tubifex	15.583	1.197
Unidentified Naidid	12.136	1.369
Phylum Arthropoda, Class Crustacea		
Subclass Branchiopoda		
Artemia	15.576	0.782
Daphnia	15.770	0.111
Scapholeberis	1.110	0.126
Subclass Ostracoda		
Unidentified Ostracod	4.721	0.082
Unidentified Ostracod	2.025	0.140
Subclass Copepoda		
Cyclopoid Copepod	5.757	0.313
Harpacticoid Copepod	0.013	0.004
Subclass Malacostraca		
Asellus	16.911	1.512
Gammarus	18.506	3.023

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