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## Aquatic Actinomycete–Fungal Interactions and Their Effects on Organic Matter Decomposition: A Microcosm Study

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### ABSTRACT

The role of fungi in the decomposition of organic matter in streams has been well examined, although the role of bacterial antagonists in such processes has gained little attention. To examine bacterial-fungal interactions, experiments involving pairwise combinations of four actinomycete isolates (*A1+* and *A2+* could remove chitin from chitin-containing media, and *A1-* and *A2-* could not) and two fungal isolates (*F+* a true fungus, *F-* an oomycete) were conducted. For each bacterial-fungal combination, 250-ml microcosms were sampled at 8 day intervals for 32 days. Microbial biomass and organic matter, as well as the activities of five extracellular enzymes, were measured. Each experiment consisted of a control group and four treatment groups. Controls comprised sterilized stream water and macrophytes. The first treatment was inoculated with only actinomycetes ( $\sim 10^3$  cells  $\text{ml}^{-1}$ ), the second treatment was inoculated with only fungi ( $\sim 10^2$  cells  $\text{ml}^{-1}$ ), the third group was inoculated simultaneously with actinomycetes and fungi, and the fourth group was inoculated with actinomycetes 2 days after fungal establishment. For all combinations, the lowest rates of organic matter decomposition were expected in the controls, as a result of only physical degradation. In contrast, the greatest rates of organic matter decomposition were predicted in treatments inoculated with *F+* 2 days prior to *A1-* or *A2-*. Greater than 50% of the organic matter was decomposed in each of the fungal treatments. Fungal–actinomycete interactions resulted in reduced fungal biomass relative to the fungal-only treatments. However, when inoculated 2 days apart, combinations of *F-* and actinomycetes resulted in enhanced rates of organic matter decomposition, as well as greater levels of extracellular enzyme activities. These results demonstrate that actinomycete–fungal interactions and their colonization dynamics affect the accumulation of biomass, extracellular enzyme activities, and rates of organic matter decomposition.

### Introduction

Efforts to understand actinomycetes and their effects on fungi have concentrated on soil-borne actinomycetes, due to

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their relatively large biomass in soils and the economic value of actinomycetes as biocontrol agents [30, 47]. To date, no studies have investigated actinomycete–fungal interactions in aquatic systems. In aquatic systems the link between microbial interactions and organic matter degradation has been

described as resource partitioning [25, 44, 45], yet studies examining specific microbial interactions are lacking.

In streams, microbial decomposition of particulate organic matter (POM) has been viewed as the procession of fungal colonization, softening organic matter through extracellular enzyme activity and physical fragmentation, followed by bacterial colonization [45]. However, the complexity of biotic interactions may alter the rates of POM decomposition. Howe and Suberkropp [23] published the first study to examine mycoparasitism on aquatic hyphomycetes, they found that *Crucella subtilis* could negatively affect early colonization and sporulation of the hyphomycete *Anguillospora filiformis*.

Aquatic microorganisms produce extracellular enzymes required for the depolymerization of organic compounds despite potential dilution [7, 38, 48]. Furthermore, aquatic fungi produce secondary metabolites that can be used in resistance of microbial infection, fungivory, and biocontrol of other fungi [18]. The production of such extracellular enzymes indicate that microbial interactions within stream habitats may have antagonistic, as well as synergistic, effects on the decomposition of organic matter.

Most studies that examine lysis of fungal cell walls by actinomycetes have focused on chitinase activity. In freshwater systems, Aumen [2] found actinomycetes to be the dominant chitinoclastic microorganism on the exoskeletons of crayfish, as well. Potential fungal–actinomycete interactions in stream systems may center on the lysis of fungal cell walls or competitive exclusion for organic carbon. Analysis of enzymes related to both chitinases, which may target fungal cell walls, and decomposition of POM will help determine primary mechanisms involved.

In this study, we examined freshwater actinomycetes and fungi in order to determine the potential direct and indirect effects of their interactions. We used two eukaryotic mycelial decomposers, one aquatic hyphomycete (chitin-containing) and one oomycete (chitin-lacking), which were paired with two actinomycetes that could clear zones of chitin from chitin-containing media and two that could not. For all interactions, microscopy was used to determine physical relationships. Since lysis by actinomycetes may be accomplished without direct colonization of the fungal mycelium [27, 29], we also examined the presence and activity of five extracellular enzymes. The extracellular enzymes screened were chosen based on their potential to initiate lysis of fungal cell walls or degrade organic matter.

The goals of this study were to determine (1) how biomass and organic matter decomposition of actinomycete–

fungal interactions differ from those of individual isolates, (2) whether fungi with chitin in their cell walls are more susceptible to antagonism by actinomycetes than those without, and (3) how initial colonization dynamics alter bacterial–fungal interactions and affect organic matter decomposition.

## Materials and Methods

### Isolates

Bacterial and fungal isolates were collected from aquatic macrophytes within Tinker Creek. Tinker Creek, which lies within the southeastern coastal plain on the U.S. Department of Energy's Savannah River Site (SRS), near Aiken Co., SC [ $33^{\circ} 22' 30''$ ,  $81^{\circ} 32' 30''$ ], is a small, low gradient, sandy-bottomed stream. The submersed aquatic macrophyte *Sparganium americanum* grows in areas of open canopy within Tinker Creek and is a good substrate from which to collect aquatic/semi-aquatic bacteria and fungi because of its anchored nature, submersed growth form, and relatively uniform shape [46].

Bacteria and fungi were isolated in the laboratory from clippings of *S. americanum*. Collected plant material was rinsed with sterile distilled water and aseptically cut into plant fragments ( $0.5 \times 1$  cm and  $0.5 \times 0.25$  cm). Plant fragments were subjected to an array of selective treatments in order to detect the greatest actinomycete diversity [46]. Plant fragments placed on selective media were then incubated for up to 35 d to allow for actinomycete growth. Whenever possible, actinomycetes were classified to genus. Initial bacterial isolates were screened based on the Gram-stain test. All Gram positive and Gram variable isolates were maintained. Taxonomic determinations were carried out on the basis of morphology using a Nikon Microphot FXA microscope in conjunction with the chemotaxonomic methods of Lechevalier and Lechevalier [26]. The identification of 20% of the distinct isolates was later confirmed by an outside agency (Microbial ID). Isolates that could not be identified to species are named/numbered according to designation numbers from the initial collection of actinomycete isolates [46]. All bacterial and fungal isolates are maintained at the University of Georgia's Savannah River Ecology Laboratory.

Eumycota were isolated by incubating plant fragments ( $0.5 \times 0.25$  cm) in a shallow layer of sterile distilled water at room temperature. After 2 d, conidia were removed from the plant fragments and transferred to 1% malt extract plates (10 g malt extract, 15 g agar, 1 L DI water). All isolates were then screened for the presence of chitin in their cell walls by epifluorescence microscopy with differential staining. The staining technique used both a chitin-specific stain, fluorescein isothiocyanate labeled lectin wheat germ agglutinin (FITC-WGA), and a fluorescent brightener, Calcofluor White (Sigma). All isolates containing chitin in their cell walls were maintained. Those isolates believed to be aquatic hyphomycetes (Deuteromycota) were then transferred to spore chambers. Spores were produced by placing fungal fragments into glass cylinders (2.5 cm diameter, 19 cm long) that had been partially filled with distilled water and fitted with stopper and air stone. Chambers were

aerated for 48 h; spores were then collected and further taxonomic identifications were made according to Ingold [24]. The hyphomycete *Anguillospora filiformis* was a common isolate from Tinker Creek and therefore was chosen for further study.

A baiting technique was used for isolation of oomycetes [10]. Plant fragments were placed in a dilute salts (Hepes K) solution with 3–4 presterilized hemp seeds. Once fungal hyphae were visibly radiating from the seeds (3–4 d), seeds were transferred to Potato Dextrose agar (Difco) with an antibiotic solution [penicillin (125 mg L<sup>-1</sup>) and streptomycin (100 mg L<sup>-1</sup>)]. Isolates were screened for chitin; those lacking chitin in their cell walls were maintained and transferred to YpG agar (1.0 g yeast extract, 20.0 g glucose, 1.0 g K<sub>2</sub>HPO<sub>4</sub>, 0.5 g MgSO<sub>4</sub> · 7H<sub>2</sub>O, 15.0 g agar, and 1 L dI water). Approximately 36 h later, 3 mm agar cubes were removed from the edge of the colonies and placed into YpG broth. After 24 h, fungal mycelia were transferred back to dilute salts solution. Microscopic examination of the sexual structures was used to confirm taxonomic identifications. An isolate of *Saprolegnia* sp. was maintained for this study.

To address our objectives, four actinomycete isolates were selected for this study based on their ability or inability to create zones of clearing on both chitin [21] and colloidal chitin [12] plates. Two actinomycete isolates of each designation were used; *Kitasatosporia griseola* and *Streptomyces* sp. 15 could not clear chitin from chitin-containing media (hereafter designated A1- and A2-, respectively), whereas *Kitasatosporia azaticus* and *Streptomyces* sp. 23 could (hereafter designated A1+ and A2+, respectively). Two fungal isolates were used in this study, one with chitin in its cell walls, *Anguillospora filiformis* (Eumycota) and one without chitin, *Saprolegnia* sp. (Oomycota) (hereafter designated F+ and F-, respectively).

### Experimental Design

The treatments were (1) control; inoculation of only sterile water, (2) bacteria only, (3) fungi only, (4) fungi and bacteria inoculated together, and (5) fungi added 2 d prior to bacterial inoculation. All possible combinations of bacteria with fungi were examined.

Studies were carried out in 250 ml Erlenmeyer flasks. Each flask was filled with 125 ml filtered (Whatman GF/F glass microfiber filter) stream water, 25 ml of 1 mM Hepes K buffer solution (pH 7.2), and six 5 cm live clippings of *Sparganium americanum*. Green tissue was used because senescent *S. americanum* tissue is rapidly decomposed and does not accumulate in these streams. However, green tissue is frequently torn from live plants during storm events and scouring by transported wood. Flasks were plugged with foam and sterilized by autoclaving (122°C for 35 min). Autoclaving ensured complete sterilization of the substrate (including propagules within the plant matter) and left no residual toxins, which can occur with fumigation, although it may have altered the composition of plant fragments [1, 40].

Actinomycetes were inoculated at densities of ~10<sup>3</sup> propagules ml<sup>-1</sup> [15, 36], whereas fungi were inoculated at densities of ~10<sup>2</sup> propagules ml<sup>-1</sup> [3, 13] to replicate densities reported in streams. To ensure proper concentration of propagules, fungal isolates were

grown on 1% malt extract plates and actinomycete isolates were grown on starch casein agar; an edge of the colony was removed (1 × 1 cm) and homogenized in a Warring blender (20 ml dI for 5 s). Propagule densities, in the form of fragmented hyphae, were estimated using direct microscopy. Each of the flasks were then inoculated and maintained on shaker tables (100 rpm) at ambient room temperature.

Using destructive sampling, four replicates of each treatment were sampled at 8 d intervals for 32 d (for a single bacterial-fungal combination: 5 treatments × 4 replicates × 5 sampling dates were established). Sampling was carried out under aseptic conditions. The 6 plant clippings were removed from the flasks; each was cut into 8 smaller sections, and these sections were then randomly assigned to plating ( $n = 2$ , to confirm the absence of contaminants), analysis of microbial biomass ( $n = 16$ ), or analysis of plant biomass ( $n = 24$ ). The remaining sections were discarded. Liquid media were centrifuged in order to concentrate mycelia not associated with plant substrate, and pH was recorded.

Estimates of cell size, biovolume and density are difficult to obtain for actinomycetes and fungi growing in plant material due to their morphology and spore production. To overcome this problem, pure cultures were used to determine an area to biomass relationship for each actinomycete isolate and *Saprolegnia* sp. An area to biomass regression equation was fitted to the data for each of the isolates from isolates grown in liquid medium (5:1 filtered stream water: Hepes K solution). At 4 d intervals, samples ( $n = 3$ ) were homogenized and filtered onto prewashed and weighed Milipore Isopore GT polycarbonate membrane filters. Filters were then dried (60°C for 3 d) and weighed using a Cahn Model 4700 Automatic electrobalance. After biomass determinations were made, filters were stained with the fluorescent brightener Calco-fluor White for microscopic examination and area determinations. Microscopic images were digitized with Mocha Image Analysis Software (Jandell Scientific). Area to biomass data were plotted for each isolate and a regression was fitted to the data. The  $r^2$  for each bacterial isolate was 0.97, 0.96, 0.96, and 0.98 for A1-, A2-, A1+, and A2+, respectively. F- had  $r^2 = 0.96$ .

Plant sections designated for analysis of bacterial biomass were stained according to Fry [14] in order to determine the area of plant tissue colonized by bacterial mycelia. Staining involved soaking the plant sections in a prestain, eosin yellow (0.2 g per L), for 1 h. Plant sections were then rinsed with distilled water and stained with phenolic aniline blue (20 ml acetic acid, 80 ml distilled water, 3.75 g phenol, 0.05 g aniline blue) for 1–2 min in order to differentiate between bacterial mycelia and plant tissues. Stained material was then examined by bright-field microscopy. Microscopic images were digitized and biomass was determined by applying the isolate-specific regression equation obtained above. Bacterial or F- mycelia not associated with plant substrate were concentrated by centrifugation, dried (60°C for 3 d), and weighed.

Standing crop biomass of F+ was determined by extracting ergosterol, a biochemical indicator of living fungal mycelia, from the fungal membrane. Plant sections ( $n = 16$ ) designated for ergosterol assays were placed in amber bottles, submersed in methanol, and stored at <4°C until they were processed. Extractions were carried

out according to Newell [34], Gessner and Newell [17], and the French Association for Normalization (AFNOR standard V18-I12). Samples were spiked with 1 ml of 7-dehydrocholesterol (10 µg per ml) to measure sample extraction efficiencies. Samples (0.05 ml) were separated by High Performance Liquid Chromatography (HPLC) on a Licosphere RP 18 (25 cm × 4.6 mm) column fitted with a Spherisorb C-18 guard column at 28°C. Flow rate of methanol solvent was 1.5 ml min<sup>-1</sup> and ergosterol and 7-dehydrocholesterol peaks were detected by 280 nm wavelength at 9.1 min and 9.7 min, respectively. A standard curve was determined using pure ergosterol and 7-dehydrocholesterol (Sigma Chemical Co.).

Hyphomycete mycelia not associated with plant material were concentrated and then visually divided in half. Each half was assigned to either dry weight determinations or ergosterol analysis. Ergosterol analysis was determined as above. Dry weight was determined by drying (60°C for 3 d) mycelia and weighing them on an electrobalance. The conversion ratio of ergosterol to biomass used for *A. filiformis* was determined to be 9.3. This finding agrees with Gessner and Chauvet [16], who report values from 6.5 to 10.1 for *A. filiformis*.

Dry weights of plant sections were measured on each sampling date, by gently rinsing the plant tissues, drying (60°C for 3 d), and weighing on an electrobalance. Sample-specific bacterial and fungal biomass estimates were then subtracted from the plant mass to determine the final organic matter dry weight.

### Enzyme Activity

To calculate enzyme activity, protein and enzyme concentrations were measured for each actinomycete–fungal combination and each of their treatments. This portion of the study was performed in 25 × 150 mm test tubes. All glassware was washed, acid rinsed, and ashed (550°C for 6 h) to remove organic residue. Each test tube was set up with the same proportions of filtered stream water (10 ml), Hepes K buffer solution (2 ml), and plant material (2–4 cm pieces) as above. Tubes were autoclaved (122°C for 35 min), inoculated accordingly, and then maintained on shaker tables (100 rpm).

Samples were taken at 0, 4, 8, 12, 16, 24, and 32 d after inoculation. On each sample date, 3 replicates of each treatment were sampled using a nonreplacement design (for each bacterial–fungal combination: 7 sampling dates × 3 replicates × 5 treatments were established). Sampling included protein assays and measurement of extracellular enzyme concentrations by use of fluorogenic substrate analogs under saturating conditions in timed assays. Protein concentration (required for determination of enzyme activity) in each of the treatments was determined by the BCA Protein Assay Reagent Kit (Pierce, Rockford, IL) with bovine serum albumin (BSA) as the standard. Absorbance (562 nm) was measured in glass cuvettes for each sample versus a water reference. Reference standards ranging from 5 to 250 µg per ml were assayed in the same manner. Standard curves were used to determine the blank corrected spectrophotometric reading for each BSA standard versus protein concentration.

Extracellular enzyme activity was measured using five fluorogenic substrate analogs. Three of the 4-methylumbelliferyl (MUF)

substrates examined allowed discrimination between β-N-acetylglucosaminidase (hydrolyzing MUF-GlcNAc) (EC 3.2.1.30), exochitinase [hydrolyzing MUF-(GlcNAc)<sub>2</sub>], and endochitinase [hydrolyzing MUF-(GlcNAc)<sub>3</sub>] (EC 3.2.1.14) [10, 31], which were glycosides of N-acetylglucosamine oligosaccharides supplied by Sigma Chemical Co., hereafter monomer, dimer, and trimer, respectively. The other two fluorogenic substrates used were MUF-β-glucopyranoside, a substrate for β-glucosidase (EC 3.2.1.21) activity, and MUF-α-mannopyranoside, a substrate for α-mannosidase (EC 3.2.1.24) activity, hereafter glucosidase and mannosidase, respectively.

Stock solutions for each of the five substrate analogs were prepared to a final concentration of 0.5 mM. Substrate analogs were first dissolved in a small amount (<1% of final volume) of Methylcellosolve (ethylene glycol monomethyl-ether), then brought to volume with sterile distilled water [22]. Stock solutions were stored at -20°C. Enzyme assays were performed using Fluoro-Nunc 96-well transparent, sterile plates (Nalge Nunc International, Naperville, IL). Wells were filled with 200 µl of vortexed treatment liquid, along with 10 µl of each fluorogenic substrate (final concentrations of MUF-substrate 2.5 nM). Plates were incubated on a shaker table (60 rpm) in the dark, at 21°C for 10 h. The reaction was terminated by the addition of 50 µl 0.5 N NaOH to each of the wells.

Fluorescence was monitored (excitation 365 nm, emission 460 nm) using a Shimadzu CS-9301PC Dual Wavelength plate reader equipped with a xenon bulb fluorometry attachment. Assays were recorded using a 1.0 × 1.0 mm beam size with a 1 sec wait time between wells. Fluorescence activities were within the linear range of the calibration curve using methylumbellifereone as the standard from which enzyme activity in nM per min per µg protein was calculated. Endogenous fluorescence was measured with “time zero” samples and subtracted from the final reading.

### Statistical Analyses

All data were normalized by dividing by the dry weight of the associated macrophyte tissue. Results are reported as ±1 standard error. Rates of decomposition were estimated using a negative exponential model using days as the time variable. Growth rate was estimated using a positive exponential model. Analysis of covariance (ANCOVA) was applied after log transformation of the biomass estimates (plant and microbes) to linearize the models [37]. The slopes resulting from these linear models are the *k*-values reported. Comparisons among treatments were made using orthogonal contrasts that compared the rates of growth or decomposition of the control to the average rates of the treatments, the bacteria alone treatments to bacteria plus fungi treatments, and the fungi alone treatments to the bacteria plus fungi treatments. In addition, each rate was tested to determine whether it was significantly different from zero (i.e., no significant growth or decomposition).

## Results

### Individual Isolates: Growth Rates and Decomposition

In treatments with only fungi, both *F+* and *F-* had growth rates and decomposition rates greater than zero (*p* < 0.0001)

**Table 1.** Rates of microbial growth for individual fungal and bacterial isolates ( $n = 4$ )<sup>a</sup>

Treatment	Bacterial growth (mg/d)	P value	Fungal growth (mg/d)	P value
<b>Fungi</b>				
<i>F</i> +	—	—	0.053	0.0001
<i>F</i> -	—	—	0.080	0.0001
<b>Bacteria</b>				
<i>A1-</i>	0.043	0.0001	—	—
<i>A1+</i>	0.026	0.0001	—	—
<i>A2-</i>	0.023	0.0001	—	—
<i>A2+</i>	0.038	0.0001	—	—

<sup>a</sup> Reported values were determined from each experiment. P value reported for rates significantly different from 0.

(Table 1). *F*+ had significantly ( $p < 0.05$  ANCOVA) faster growth rates than *F*- (0.080 and 0.053, respectively) whereas the rate of decomposition was nearly four times faster ( $p < 0.05$ ) in the *F*+ (−0.056) treatments than the *F*- (−0.019). Maximum biomass occurred much earlier for *F*+ than *F*- (Fig. 1). Plant decomposition by *F*- was not different from the bacterial isolates (Fig. 2). Among the actinomycetes, the fastest growth rates were observed for *A1-* (Table 1), but because of high variability only *A2+* had decomposition rates significantly different from zero. All bacterial isolates had obtained peak biomass (Fig. 1) and greatest loss of plant tissue (Fig. 2) at approximately day 8.

#### Actinomycete and Fungal Interactions: Growth Rates

Growth rates of *F*+ inoculated with actinomycetes (Table 2) were significantly slower than growth rates of individual *F*+ isolates (Table 1). In some cases (i.e., *A1+* and *A2+* with *F*), accumulation of *F*+ biomass was not significantly different from zero, suggesting that these bacteria may be controlling the *F*+ growth. Growth rates of the chitin containing fungi *F*+ inoculated 2 days prior to bacterial introduction were never different from zero (Table 2). Growth rates of the actinomycetes inoculated along with *F*+ (Table 2) were similar to the rates for the individual isolates (Table 1) with the exception of *A1+*, which was depressed. When the bacteria were introduced two days after *F*+ inoculation, all strains except *A1-* had higher growth rates than the respective individual isolates (Tables 1 and 2).

The effect of simultaneous inoculation of actinomycetes with *F*- growth was similar among treatments and resulted in no measurable growth of the fungi for any combination of bacteria (Table 3). When the bacteria were introduced 2 days

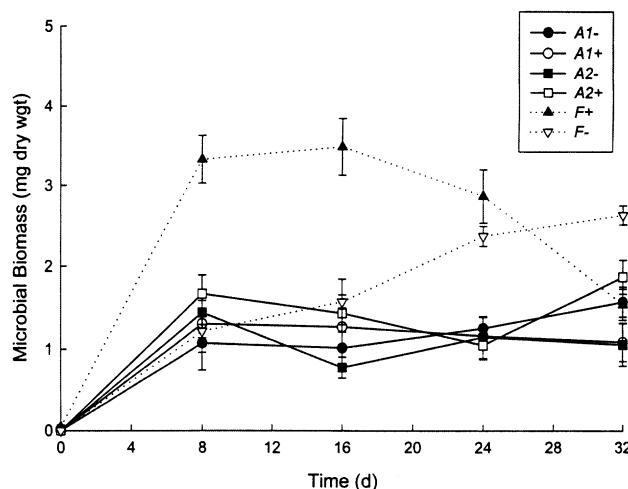


Fig. 1. Microbial biomass (mg dry wgt  $\pm 1$  SE) of each microbial isolate on each sampling date. Absence of standard error bars for some samples is a result of their close proximity to the reported mean.

after the fungi (Table 3), *F*- growth was also depressed relative to individual isolates of *F*- (Table 1). Growth rates of the actinomycetes when simultaneously or separately inoculated with *F*- (Table 3) were slightly higher or similar to rates for the individual isolates (Table 1).

#### Actinomycete and Fungal Interactions: Decomposition

When fungi and bacteria were inoculated simultaneously, treatments with *F*+ always had faster rates of decomposition

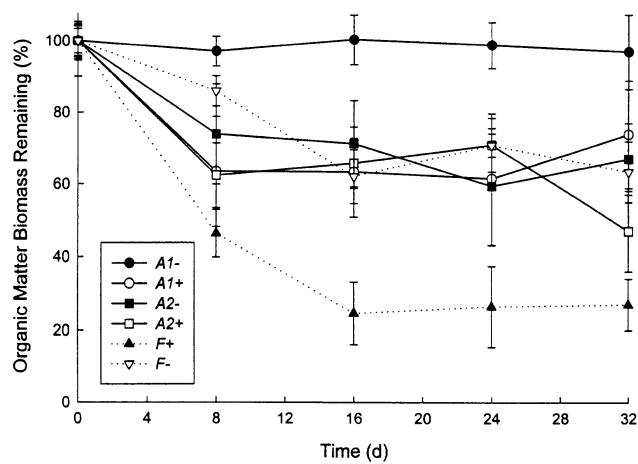


Fig. 2. Percent remaining of particulate organic matter biomass ( $\pm 1$  SE) for each microbial isolate on each sampling date. Absence of standard error bars for some samples is a result of their close proximity to the reported mean.

**Table 2.** Rates of microbial growth and organic matter decomposition for bacterial-fungal treatments inoculated with *F+* (*A. filiformis*) and one of four actinomycetes ( $n = 4$ )<sup>a</sup>

Treatment	Bacterial growth (mg/d)	P value	Fungal growth (mg/d)	P value
Simultaneous treatment				
<i>F+/A1-</i>	0.060	0.0001	0.012	0.050
<i>F+/A1+</i>	0.013	NS	0.004	NS
<i>F+/A2-</i>	0.028	0.0015	0.015	0.050
<i>F+/A2+</i>	0.054	0.001	0.003	NS
Treatment 2 days apart				
<i>F+/A1-</i>	0.010	NS	0.011	NS
<i>F+/A1+</i>	0.033	0.0052	0.0009	NS
<i>F+/A2-</i>	0.036	0.0001	0.007	NS
<i>F+/A2+</i>	0.041	0.012	0.001	NS

<sup>a</sup> Reported values were determined individually for each bacterial-fungal combination. P value reported for rates significantly different from 0.

than treatments with *F-*, regardless of which actinomycete was used (Fig. 3A). Fastest rates of decomposition were obtained when *A2+* was inoculated with *F+* ( $k = -0.024$ ). Slowest decomposition ( $k = -0.004$ ) occurred when *A1+* was inoculated with *F-*. In contrast, when the fungi were inoculated two days prior to bacterial inoculation, *F-* had significantly greater rates of decomposition (Fig. 3B) than *F+* in three of the four combinations. These decomposition rates were nearly four times the rates of *F-* when inoculated simultaneously with bacteria.

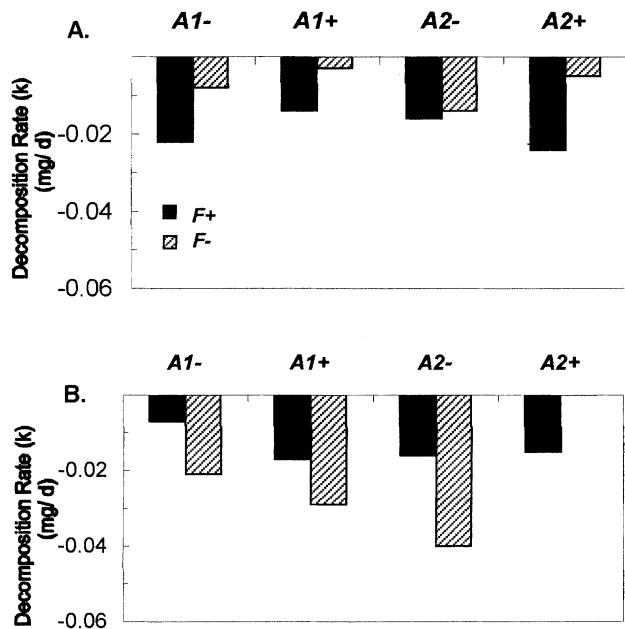
#### Individual Isolates: Enzymes

Glucosidase and mannosidase differed between the two fungal isolates (Fig. 4). In the case of *F-*, glucosidase activity

**Table 3.** Rates of bacterial and fungal growth as well as organic matter decomposition for treatments inoculated with *F-* (*Saprolegnia* sp.) and one of four actinomycetes ( $n = 4$ )<sup>a</sup>

Treatment	Bacterial growth (mg/d)	P value	Fungal growth (mg/d)	P value
Simultaneous treatment				
<i>F-/A1-</i>	0.047	0.0001	0.002	NS
<i>F-/A1+</i>	0.031	0.0001	0.0001	NS
<i>F-/A2-</i>	0.024	0.018	0.001	NS
<i>F-/A2+</i>	0.014	NS	0.0001	NS
Treatment 2 days apart				
<i>F-/A1-</i>	0.067	0.0001	0.010	NS
<i>F-/A1+</i>	0.053	0.0001	0.012	0.050
<i>F-/A2-</i>	0.020	0.044	0.006	NS
<i>F-/A2+</i>	0.030	0.018	0.0000	NS

<sup>a</sup> Reported values were determined individually for each bacterial-fungal combination. P value reported for rates significantly different from 0.



**Fig. 3.** Comparison of decomposition rates ( $k$ ) of plant biomass per day ( $\text{mg d}^{-1}$ ) for each actinomycete-fungal interaction. (A) Simultaneous inoculation of actinomycetes and fungi. (B) Fungi inoculated 2 days prior to actinomycetes.

increased between day 4 and 8, maintaining a relatively constant level of activity thereafter. Overall, *F+* had significantly more glucosidase activity than *F-* ( $P < 0.0001$ ). Similarly, mannosidase activity was greater for *F+* than *F-* on day 16 ( $P < 0.0001$ ). *F-* had no notable chitin-related enzyme activity (Fig. 5), while *F+* monomer, dimer, and trimer activity peaked on day 24, as fungal biomass started to decline (Fig. 5).

In individual bacterial treatments with *A1-*, *A1+*, or *A2+*, both glucosidase and mannosidase activities were low (Fig. 4). *A2-* had significantly greater glucosidase activity on each sampling date ( $P < 0.0001$ ) than the other bacterial isolates, as well as notably more monomer than the other actinomycetes (Fig. 5A).

#### Actinomycete and Fungal Interactions: Enzymes

To further determine whether actinomycetes had a positive or negative effect on fungi, we examined enzyme activity of bacteria and fungi alone and under the treatments imposed. We then added the values obtained singly for each bacterial isolate and each fungal isolate together and divided this number by the enzyme activity found when bacteria and fungi were grown together to obtain a proportion. If this value was at or near 1.0, then there was no interaction be-

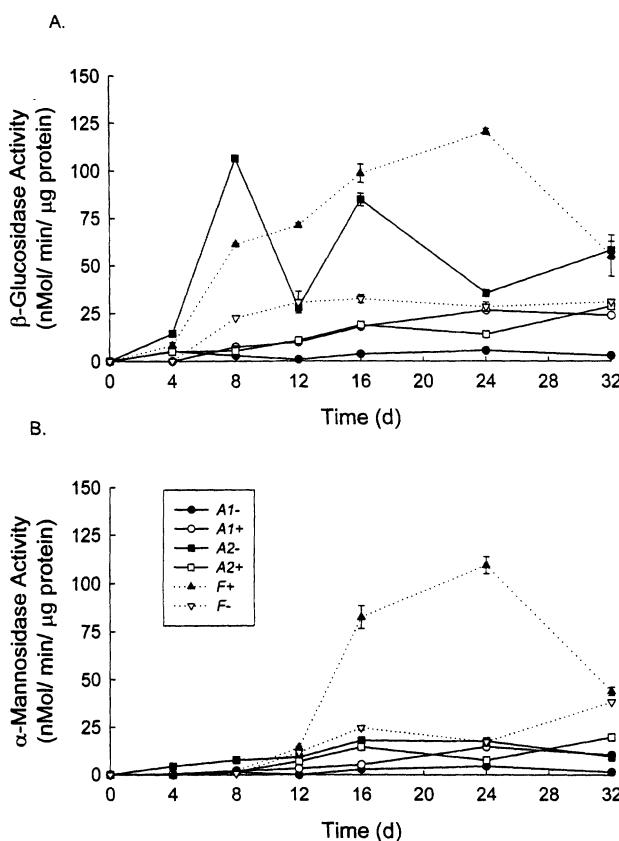


Fig. 4. Comparison of enzyme activities ( $\text{nmol min}^{-1} \mu\text{g protein}^{-1}$ ) on substrate analogs per sampling date for individual bacterial and fungal isolates. Error bars represent 1 SE. Absence of standard error bars for some samples is a result of their close proximity to the mean. (A) Glucosidase activity. (B) Mannosidase activity

tween the bacteria and fungi. Values above 1.0 indicated greater enzyme production by individual isolates than the interactive counterparts, while values below 1.0 suggested greater enzyme production together than alone. These values were calculated for all possible combinations and for all five enzymes examined (Figs. 6 and 7).

Generally enzymatic activity was increased when  $A1+$  and  $A1-$  were grown simultaneously with either  $F+$  or  $F-$  (Fig. 6). However, with the exception of glucosidase activity, adding  $A2+$  and  $A2-$  with the fungi resulted in lower or neutral enzymatic activity, especially for  $F+$  combinations. This was especially true for dimer and trimer activities.

The effect of inoculating the bacteria two days after fungi on enzymatic activity was similar to that found with the simultaneous inoculation for  $A1+$  and  $A1-$  (Fig. 7). There was some indication that glucosidase and trimer activity was reduced when  $A1-$  was present with  $F-$ . Treatment combinations with  $A2-$  and  $F-$  showed reduced activity for glu-

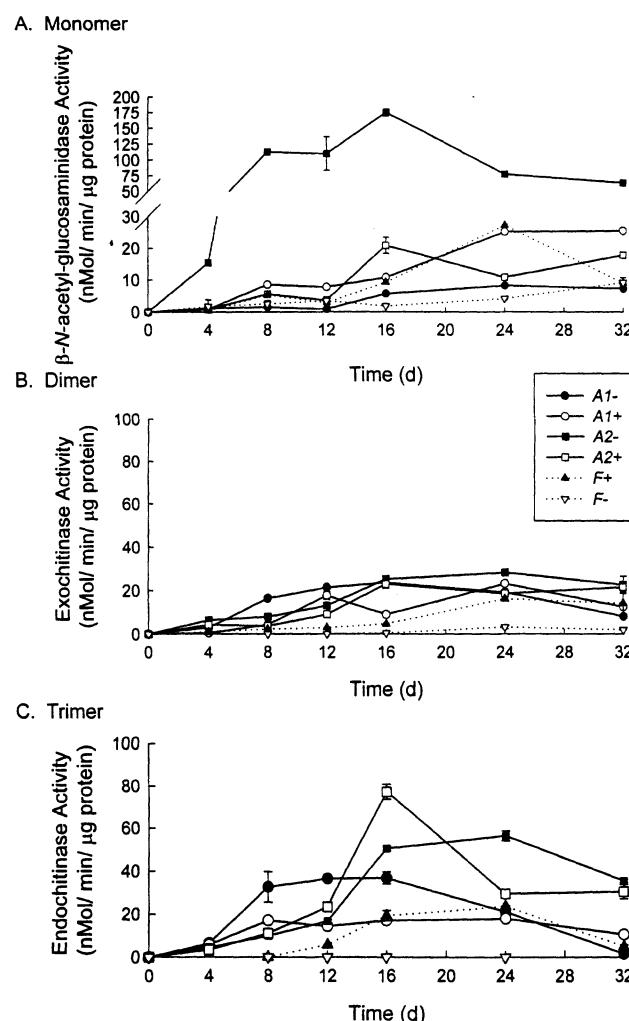


Fig. 5. Comparison of chitin-related enzyme activities ( $\text{nmol min}^{-1} \mu\text{g protein}^{-1}$ ) on substrate analogs per sampling date for individual bacterial and fungal isolates. Error bars represent 1 SE. Absence of standard error bars for some samples is a result of their close proximity to the mean. (A) Monomer. (B) Dimer. (C) Trimer.

cosidase and mannosidase, whereas glucosidase, monomer, and especially mannosidase activity was reduced when  $A2+$  was grown with  $F-$ .

## Discussion

### Actinomycete–Fungal Interactions

We have shown that actinomycete-fungal interactions in aquatic microcosms have the potential to be antagonistic whether or not chitin is present. All four actinomycetes exhibited similar growth rates, growing most rapidly between day 0 and 8. Such rapid growth may have been due to the

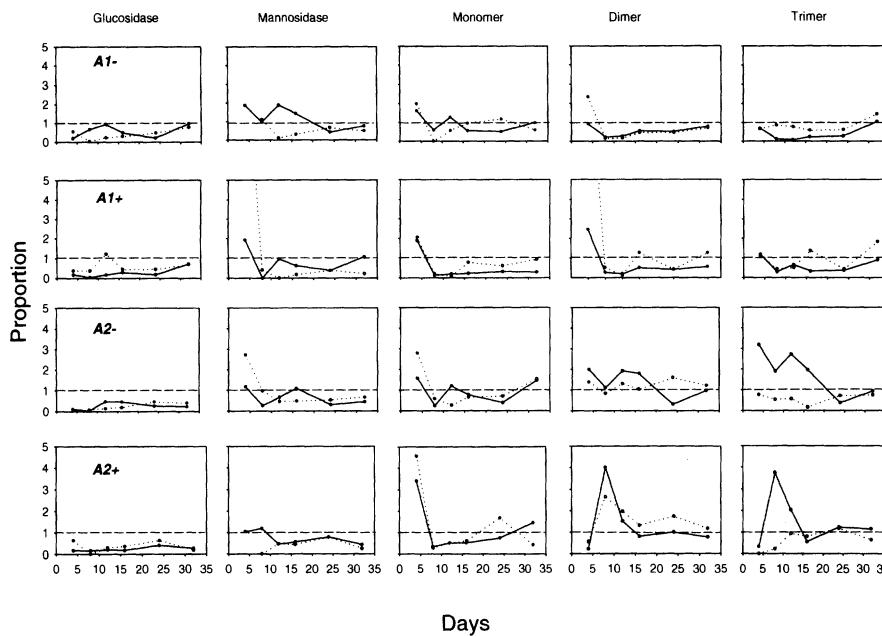


Fig. 6. Simultaneous inoculation. Enzyme activities represented as a proportion of the addition of individual enzyme concentrations of the two isolates ( $F/A$ ) obtained under individual treatments divided by the enzyme activity obtained when bacteria and fungi were grown together. Solid circles (●) indicate  $F+$ ; hollow circles (○) indicate  $F-$ .

initial availability of dissolved and very fine particulate organic matter. Despite the intrusive nature of filamentous growth by actinomycetes, only  $A2+$  significantly degraded particulate organic matter (POM). Growth of the other actinomycetes may be attributable to consumption of dissolved organic materials with additional nutrient enrichment from consumption of older substrate mycelia [9].

Each fungal isolate gained significantly more biomass than the actinomycetes.  $F+$  had the greatest growth rate between day 0 and 8, whereas  $F-$  increased steadily through-

out the study.  $F+$  decomposed more than 72% of available organic matter, while  $F-$  decomposed 53%. This finding suggests that, unlike actinomycetes, both fungal isolates acquire nutrients from POM in these microcosms, which agrees with observations of others [4, 35, 43].

We determined that actinomycete-fungal interactions may either be directly (e.g., competition for primary resource capture) or indirectly (e.g., lytic agents, antibiotics) antagonistic, resulting in reduced fungal growth. No physical attachment between bacteria and fungi was apparent by mi-

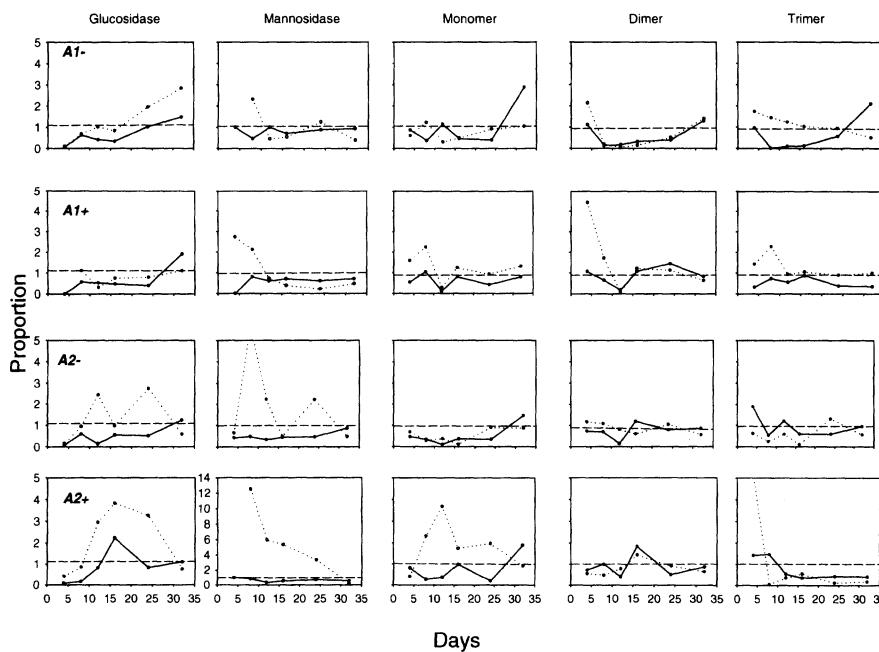


Fig. 7. Inoculation 2 days apart. Enzyme activities represented as a proportion of the addition of individual enzyme concentrations of the two isolates ( $F/A$ ) obtained under individual treatments divided by the enzyme activity obtained when bacteria and fungi were grown together. Solid circles (●) indicate  $F+$ ; hollow circles (○) indicate  $F-$ .

croscopic examination of POM during the study. However, actinomycetes can inhibit fungal activity without direct contact (e.g., fungicides, extracellular enzymes, or other compounds) [11, 19, 27, 28].

Simultaneous inoculation of fungi and bacteria yielded significantly reduced fungal growth, while bacterial biomass was similar to respective bacteria treatments. Neither *F+* nor *F-*, when grown with bacteria, ever accumulated more than 1 mg dry wgt, whereas individually they were at least 3 times greater, demonstrating that actinomycetes can inhibit fungi. Although spore presence was not differentiated from fungal biomass, spores may have been a good indicator of stress for each of the fungal isolates. Reduced fungal growth by actinomycetes was most likely due to the presence of an extracellular compound (other than chitinases), competition for colonization substrata, or direct competition for a trace element/nutrient in the water filtrate. Direct competition for organic matter and chitinases was dismissed, since single isolate treatments indicated primary use of POM by fungi and dissolved organic matter by actinomycetes with similar negative effects on both fungal isolates.

When bacteria and fungi were inoculated simultaneously, treatments with *F+* had faster rates of decomposition than treatments with *F-* (Fig. 3). The fastest rate obtained under the simultaneous inoculation was *A2+/F+*. *A priori* we would have expected this combination to be among the slowest, since an actinomycete that could clear chitin was grown with a fungus containing chitin. Interestingly, when bacteria were inoculated two days after fungi, the presence of bacteria reduced decomposition of *F+* treatments (Fig. 3). We had anticipated that allowing the fungi 2 days to establish would decrease the bacteria's potential negative affect. This was not observed. Furthermore, in *F-* treatments rates of decomposition were greatly enhanced when bacterial inoculations were delayed by 2 days. Clearly these combinations had a synergistic effect on decomposition.

Enzyme activities were measured to help establish possible mechanisms involved in bacterial-fungal interactions. Monomer, dimer, and trimer activities by the fungal isolates (Fig. 5) agreed with previously reported findings that all true fungi examined to date produce chitinases for apical extension of fungal cell walls, whereas oomycetes do not [5, 20]. Enzyme activities (Fig. 5) of the actinomycetes indicates that chitinolytic abilities, based on cleared zones of chitin, were inaccurate. Zare-Maivan and Shearer [48], as well as Chamiér [7], similarly found that agar plate assays underestimated the number of fungal species capable of degrading chitin. Incongruence in enzymic abilities may result from

differences in culture conditions, which do not induce chitinase production [7, 48]. Other factors may include an inability for actinomycetes to uptake the monomeric form,  $\beta$ -N-acetylglucosamine, of chitin. Although this may have been true for *A1-*, which had low monomer activity, it does not explain the results of *A2-*, which had relatively high levels of monomer activity. Alternatively, extracellular chitinase-related activity by actinomycetes observed in the experiments may have occurred from excretion of spore-bound chitinases used to release their spores, rather than to consume exogenous chitin [41, 42].

As noted, the aquatic hyphomycete was no more susceptible to antagonism by actinomycetes than the oomycete. Earlier studies found that densities of actinomycetes increased after amending soil with chitin or living fungal mycelia [32, 33, 39]. Moreover, Aumen [2] found that actinomycetes were dominant chitinoclastic organisms in streams using crayfish exoskeletons as bait. Some of the microcosms containing bacteria and fungi had enhanced extracellular enzymic activity (Figs. 6 and 7). In all simultaneous inoculations of *F+* with actinomycetes, relatively high levels of decomposition were observed along with positive combined enzyme activity effects. This was particularly evident with *A2+* and *F+*, where mannosidase activity was enhanced. Staggered colonization of actinomycetes with *F-* yielded greater rates of decomposition despite reduction in overall enzyme production. Other enzymes not examined here may have been better indicators of organic matter decomposition or potential bacterial-fungal interactions. Findings by Beyers and Diekmann [6] indicate that filamentous fungal cell walls each require a fungal-specific consortium of enzymes, including those that target wall-bound lipids and proteins, which suggests that analysis of more than five enzymes may have aided in interpretation of results.

### Colonization Dynamics

In a stream environment, it is unlikely that bacterial and fungal spores always encounter substrata simultaneously. Inoculation of bacteria two days after fungi simulated staggered colonization of organic matter. Greater rates of decomposition are predicted when fungi are primary colonizers. Although fungi may increase decomposition rates by excretion of degradative enzymes and physical fragmentation [8, 44], our findings indicate that this relationship is not always positive. While *in situ* studies are needed, the observed antagonistic interactions may indirectly enhance POM decomposition by increasing the rate of sporulation by

*F+*, subsequently resulting in greater dissemination of spores. However, such antagonistic interactions may also result in decreased rates of POM decomposition by maintaining a lower standing crop of fungal biomass.

Despite suppression of fungal biomass by actinomycetes, delayed bacterial growth was also observed. For those actinomycete-fungal interactions with delayed bacterial growth, a lag time of 16 to 24 days was required for bacteria to establish levels of biomass comparable to the respective bacteria treatments. The delay in bacterial growth indicates that despite the antagonistic effect of actinomycetes on fungi, fungi have some ability to inhibit actinomycetes. Suberkropp and Klug [44] stated that fungi in temperate streams must first condition particulate matter before an increase in bacteria is observed. Although fungal conditioning may be required in some cases, it was not necessary for these particular actinomycetes, substantiated by greater bacterial growth in the absence of fungi. It appears, however, that delayed bacterial growth was due to an inhibitive property of fungi, which has also been observed by Chamier et al. [8]. Fungal properties that may inhibit bacteria included eliminating suitable colonization substrata, absence of essential nutrients (which had already been consumed by the fungi), or compounds secreted by the fungi.

In the 2 days apart *F-* treatments, there was greater *F-* biomass than in the respective simultaneous treatments, although both *F-* and the actinomycetes had reduced biomass relative to individual treatments. However, their combined effect on organic matter was enhanced (with the exception of A2-). This apparent synergistic effect on POM decomposition was unexpected. These rates of POM decomposition were almost double those of individual isolates. Levels of both glucosidase and mannosidase activities were also high in these treatments, suggesting that time of colonization may affect consumption of cellulose and other recalcitrant compounds. Although the mechanisms remain unclear, it may be attributed to induced enzyme production or removal of inhibitory compounds by one of the organisms. Alternatively, POM degradation may be enhanced through the combined extracellular activity of fungi and bacteria.

## Conclusions

Microorganisms can affect ecosystem processes, yet it is often difficult to interpret the dynamics that govern involved microbial interactions. Microcosm studies are valuable in determining the potential direct and indirect effects that

simple microbial interactions have within larger frameworks. The examination of eight bacterial-fungal combinations demonstrated that antagonistic, as well as synergistic, actinomycete-fungal interactions can occur under laboratory conditions. The results further demonstrated that antagonism of fungi by certain actinomycetes occurred whether chitin was present or absent in the fungal cells. More *in situ* work is needed to understand these complex interactions, yet this study demonstrates that actinomycetes in aquatic systems can inhibit fungal growth and indirectly alter rates of POM decomposition.

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## References

1. Aldrich DG, Martin JP (1952) Effect of fumigation on some chemical properties of soils. Paper No. 703, University of California Citrus Experiment Station. Soil Sci 73:149–159
2. Aumen NG (1980) Microbial succession on a chitinous substrate in a woodland stream. Microb Ecol 6:317–327
3. Bärlocher F (1992) Effects of drying and freezing autumn leaves on leaching and colonization by aquatic hyphomycetes. Freshwat Biol 28:1–7
4. Bärlocher F, Kendrick B (1981) Role of aquatic hyphomycetes in the trophic structure of streams. In: Wicklow DT, Carroll GG (eds) The Fungal Community: Its Organization and Role in the Ecosystem. Marcel Dekker, New York, pp 743–760
5. Bartnicki-Garcia S (1973) Fundamental aspects of hyphal morphogenesis. In: Ashworth JM, Smith JE (eds) Microbial Differentiation. Cambridge University Press, New York, pp 245–267
6. Beyers M, Diekmann H (1985) The chitinase system of *Streptomyces* sp. ATCC 11238 and its significance for fungal cell wall degradation. Appl Microbiol Biotechnol 23:140–146
7. Chamier A-C (1985) Cell-wall-degrading enzymes of aquatic hyphomycetes: A review. Bot J Linnean Soc 91:67–81
8. Chamier A-C, Dixon PA, Archer SA (1984) The spatial distribution of fungi on decomposing alder leaves in a freshwater stream. Oecologia 64:92–103

9. Chater KF, Losick R (1997) Mycelial life style of *Streptomyces coelicolor* A3(2) and its relatives. In: Shapiro JA, Dworkin M (eds) *Bacteria as Multicellular Organisms*. Oxford University Press, New York, pp 149–182
10. Chernin L, Ismailov Z, Haran S, Chet I (1995) Chitinolytic *Enterobacter agglomerans* antagonistic to fungal plant pathogens. *Appl Environ Microbiol* 61:1720–1726
11. Crawford DL, Lynch JM, Whippes JM, Ousley MA (1993) Isolation and characterization of actinomycete antagonists of a fungal root pathogen. *Appl Environ Microbiol* 59:3899–3905
12. Cross T, Attwell RW (1974) Recovery of viable thermoactinomycete endospores from deep mud cores. In: Barker AN, Gould GW, Wolf J (eds) *Spore Research*. Academic Press, London, pp 11–20
13. Dix NJ, Webster J (1995) Aquatic fungi. In: Dix NJ, Webster J (eds) *Fungal Ecology*. Chapman & Hall, New York, pp 225–283
14. Fry JC (1988) Determination of biomass. In: Austin B (ed) *Methods in Aquatic Bacteriology*. John Wiley & Sons, New York, pp 27–72
15. Gavrilova NA (1982) Distribution of Actinomycetes in natural waters (A survey). *J Hydrobiol* 1:24–31
16. Gessner MO, Chauvet E (1993) Ergosterol-to-biomass conversion factors for aquatic hyphomycetes. *Appl Environ Microbiol* 59:502–507
17. Gessner MO, Newell SY (1997) Bulk quantitative methods of the examination of eukaryotic organoosmotrophs. In: Hurst CJ, Knudson G, McInerney M, Stetzebach SL, Winter M (eds) *Manual of Environmental Microbiology*. ASM Press, Washington, DC, pp 295–308
18. Gloer JB (1995) The chemistry of fungal antagonism and defense. *Can J Bot* 73(Suppl. 1):S1265–S1274
19. Griffin GJ (1962) Production of a fungistatic effect by soil microflora in autoclaved soil. *Phytopathology* 52:90–91
20. Hodge A, Alexander IJ, Gooday GW (1995) Chitinolytic enzymes of pathogenic and ectomycorrhizal fungi. *Mycol Res* 99:935–941
21. Hood EG, Meyers K (1977) Rates of chitin degradation in an estuarine environment. *J Oceanogr Soc Japan* 33:328–334
22. Hoppe HG (1983) Significance of exoenzymatic activities in the ecology of brackish water: Measurements by means of methylumbelliferyl-substrates. *Mar Ecol Prog Ser* 11:299–308
23. Howe MJ, Suberkropp K (1993) Effects of mycoparasitism on an aquatic hyphomycete growing on leaf litter. *Mycologia* 85:898–901
24. Ingold CT (1975) An illustrated guide to aquatic and water-borne hyphomycetes (Fungi Imperfetti) with notes on their biology. Scientific Publication No. 30. Freshwater Biological Association, Ambleside, PA, p 96
25. Kaushik NK, Hynes HBN (1971) The fate of the dead leaves that fall into streams. *Arch Hydrobiol* 68:465–515
26. Lechevalier MP, Lechevalier HA (1980) The chemotaxonomy of actinomycetes. In: Dietz A, Thayer DW (eds) *Actinomycete Taxonomy. Procedures for Studying Aerobic Actinomycetes* with Emphasis on the Streptomycetes. Society for Industrial Microbiology, Washington, DC, pp 227–291
27. Lloyd AB, Lockwood JL (1966) Lysis of fungal hyphae in soil and its possible relation to autolysis. *Phytopathology* 56:595–602
28. Lockwood JL (1960) Lysis of mycelium of plant-pathogenic fungi by natural soil. *Phytopathology* 50:787–789
29. Lockwood JL (1968) The fungal environment of soil bacteria. In: Gray TRG, Parkinson D (eds) *The Ecology of Soil Bacteria*. University of Toronto Press, Toronto, pp 44–65
30. Lumsden RD (1992) Mycoparasitism of soilborne plant pathogens. In: Wicklow DT, Carroll GG (eds) *The Fungal Community: Its Organization and Role in the Ecosystem*. Marcel Dekker, New York, pp 275–293
31. McCreath KJ, Gooday GW (1992) A rapid and sensitive microassay for determination of chitinolytic activity. *J Microbiol Meth* 14:229–237
32. Mitchell R (1963) Addition of fungal cell-wall components to soil for biological disease control. *Phytopathology* 53:1068–1071
33. Mitchell R, Alexander M (1962) Microbiological processes associated with the use of chitin for biological control. *Soil Sci Soc Amer Proc* 26:556–558
34. Newell SY (1993) Membrane-containing fungal mass and fungal specific growth rate in natural samples. In: Kemp PF, Sherr FB, Sherr EF, Cole JJ (eds) *Handbook of Methods in Aquatic Microbial Ecology*. Lewis Publishers, Boca Raton, FL, pp 579–586
35. Newell SY, Fell JW (1997) Competition among mangrove oomycetes, and between oomycetes and other microbes. *Aquat Microb Ecol* 12:21–28
36. Raschke RL, Carroll B, Tebo LB (1975) The relationship between substrate content, water quality, actinomycetes and musty odours in the Broad River Basin. *J Appl Ecol* 12:535–560
37. SAS Institute, Inc. (1996) *SAS Release 6.12*. Cary, NC
38. Sinsabaugh RL, Findlay S (1995) Microbial production, enzyme activity, and carbon turnover in surface sediments of the Hudson River estuary. *Microb Ecol* 30:127–141
39. Skinner FA (1956) The effect of adding clays to mixed cultures of *Streptomyces albidoflavus* and *Fusarium culmorum*. *J Gen Microbiol* 14:393–405
40. Skipper HD, Westermann DT (1973) Comparative effects of propylene oxide, sodium azide, and autoclaving on selected soil properties. *Soil Biol Biochem* 5:409–414
41. Smucker RA (1984) Biochemistry of *Streptomyces* spore sheath. In: Ortiz-Ortiz L, Bojalil LF, Yakoleff V (eds) *Biological, Biochemical, and Biomedical Aspects of Actinomycetes*. Academic Press, Orlando, FL, pp 171–177
42. Smucker RA, Morin LG (1985) *Streptomyces* sp. chitin development during sporulation. In: Szabo G, Biro S, Goodfellow M (eds) *Sixth International Symposium on Actinomycetes Biology*. Kiado Academic Press, Budapest, pp 465–473
43. Suberkropp K (1992) Aquatic hyphomycete communities. In: Wicklow DT, Carroll GG (eds) *The Fungal Community: Its*

- Organization and Role in the Ecosystem. Marcel Dekker, New York, pp 729–747
- 44. Suberkropp K, Klug MJ (1976) Fungi and bacteria associated with leaves during processing in a woodland stream. Ecology 57:707–719
  - 45. Webster JR, Benfield EF (1986) Vascular plant breakdown in freshwater ecosystems. Ann Rev Ecol Syst 17:567–594
  - 46. Wohl DL, McArthur JV (1998) Actinomycete-flora associated with submersed freshwater macrophytes. FEMS Microbiol Ecol 26:135–140 DOI: 10.1016/S0168-6496(98)00029-4
  - 47. Yuan WM, Crawford DL (1995) Characterization of *Streptomyces lydicus* WYEC108 as a potential biocontrol agent against fungal root and seed rots. Appl Environ Microbiol 61:3119–3128
  - 48. Zare-Maivan H, Shearer CA (1988) Extracellular enzyme production and cell wall degradation by freshwater lignicolous fungi. Mycologia 80:365–375