Applied and Environmental Microbiology, Feb. 2011, p. 803–809 Vol. 77, No. 3
0099-2240/11/$12.00 doi:10.1128/AEM.01527-10

Experimentally Simulated Global Warming and Nitrogen Enrichment Effects on Microbial Litter Decomposers in a Marsh[▽]

Sabine Flury[1,2]* and Mark O. Gessner[1,2]†

Department of Aquatic Ecology, Eawag, Swiss Federal Institute of Aquatic Science & Technology, Überlandstrasse 133, 8600 Dübendorf, Switzerland,[1] and Institute of Integrative Biology (IBZ), ETH Zurich, Zurich, Switzerland[2]

Received 28 June 2010/Accepted 1 December 2010

Atmospheric warming and increased nitrogen deposition can lead to changes of microbial communities with possible consequences for biogeochemical processes. We used an enclosure facility in a freshwater marsh to assess the effects on microbes associated with decomposing plant litter under conditions of simulated climate warming and pulsed nitrogen supply. Standard batches of litter were placed in coarse-mesh and fine-mesh bags and submerged in a series of heated, nitrogen-enriched, and control enclosures. They were retrieved later and analyzed for a range of microbial parameters. Fingerprinting profiles obtained by denaturing gradient gel electrophoresis (DGGE) indicated that simulated global warming induced a shift in bacterial community structure. In addition, warming reduced fungal biomass, whereas bacterial biomass was unaffected. The mesh size of the litter bags and sampling date also had an influence on bacterial community structure, with the apparent number of dominant genotypes increasing from spring to summer. Microbial respiration was unaffected by any treatment, and nitrogen enrichment had no clear effect on any of the microbial parameters considered. Overall, these results suggest that microbes associated with decomposing plant litter in nutrient-rich freshwater marshes are resistant to extra nitrogen supplies but are likely to respond to temperature increases projected for this century.

Microbes are crucial drivers of most biogeochemical transformations in the biosphere. As the climate is warming (31) and anthropogenic nitrogen deposition continues (19), their habitat conditions change, and this is likely to cause shifts in species distributions and novel microbial communities (28). Altered communities can in turn change rates of biogeochemical processes and other ecosystem properties (5, 25). A key question is, therefore, to what extent microbial communities will be influenced by global environmental change and how ecosystems will be affected through corresponding changes in microbial activities. Experiments that manipulate climate conditions and other environmental factors are a powerful means to assess global change effects on microbes and their activities. These experiments are likely to yield the most realistic results when conducted in natural environments. To date, however, experiments simulating global environmental changes under field conditions have rarely (e.g., see reference 30) provided information on microbial responses.

One of the most important biogeochemical processes involving microbes is the decomposition of litter produced by vascular plants (25, 38). Well known in forests and grasslands, litter decomposition is also a vital process in many aquatic ecosystems (48). This includes wetlands dominated by emergent aquatic vegetation (21) where plant production often is high (1.5 kg shoot dry mass m^{-2} $year^{-1}$ or more [23, 29]), and only a small fraction is consumed by herbivores (11). In these systems, both bacteria and fungi colonize plant litter and produce a substantial biomass and/or play an important role in the decomposition process (9, 21, 36, 41). The microbial communities colonizing decomposing plant litter can be characterized by means of bulk measures such as total bacterial and fungal biomass and by activity measurements such as respiration, which provide a broad integrative assessment of aerobic microbial metabolism (e.g., see reference 26). In addition, microbial communities can be described in terms of community structure. One of the standard approaches for this purpose is denaturing gradient gel electrophoresis (DGGE), which produces fingerprint profiles by separating PCR-generated DNA fragments according to their sequence (14, 39, 42). Although DGGE has some limitations (like all PCR-based techniques), the community profiles generated by the method have proved most valuable to describe microbial communities in various environments (42), including decomposing plant litter in wetlands (6, 8). Since the methodological biases are likely to be similar in a given sample matrix, the approach is particularly effective for detecting changes occurring in response to experimental manipulations of environmental conditions (42).

The aim of the present study was to assess the response of aquatic microbes associated with decomposing plant litter under the influence of climate warming and nitrogen enrichment. To this end, we manipulated temperature and nitrogen supply in an enclosure experiment and determined effects on bacterial and fungal biomass, total microbial respiration, and potential shifts in bacterial community structure associated with decomposing leaf litter. Given the importance of temperature and nutrient supply for microorganisms, we hypothesized that both factors would lead to clear qualitative (community structure)

* Corresponding author. Present address: Center for Geomicrobiology, Department of Biological Sciences, Aarhus University, Ny Munkegade 114-116, Bldg. 1540, 8000 Aarhus C, Denmark. Phone: 45 8942 3275. Fax: 45 8942 2722. E-mail: sabine.flury@biology.au.dk.

† Present address: Leibniz Institute of Freshwater Ecology and Inland Fisheries (IGB) and Institute of Ecology, Berlin Institute of Technology (TU Berlin), Alte Fischerhütte 2, 16775 Stechlin, Germany.

▽ Published ahead of print on 10 December 2010.

and quantitative (biomass and respiration activity) changes. In addition, we aimed to assess the influence of macroinvertebrates on the microbial communities by enclosing litter in mesh bags with large and small mesh sizes to allow or prevent feeding on the litter and associated microbes by detritivores.

MATERIALS AND METHODS

Study site. An experiment simulating global warming and chronic anthropogenic nitrogen deposition was performed in a series of enclosures established in a littoral freshwater marsh. The site was located on the eastern shore of a eutrophic postglacial moraine lake in central Switzerland (Lake Hallwil; 46°54′ N; 6°54′ E) at 449 m above sea level. The lake has a surface area of 10.2 km^2, a water volume of 0.292 km^3, and an average and maximum depth of 29 and 48 m, respectively. Vegetation in the marsh was composed mainly of common reed, *Phragmites australis* (Cav.) Trin. ex Steud., whose shoots extended up to 130 cm below the water surface and up to 3 m above the water level. Sediment in the marsh was permanently submerged, since seasonal fluctuations of the water level were buffered by a weir at the lake outlet. The average width of the reed stand was about 10 m. Wave action was strongly damped within the reed stand.

Experimental setup. The experiment was performed using a randomized block design, with each of four blocks in the marsh consisting of four enclosures plus an unfenced control plot of equal size. Two enclosures in each block were electrically heated to raise the temperature to a target of 4°C above the ambient water temperature. This treatment will be referred to as H. The two other enclosures per block (designated 0) and the unfenced lake control plot (L) were kept at ambient water temperature. Nitrate was added to one of the heated and one of the ambient-temperature enclosures (N). The comparison of the unheated and unenriched control mesocosms to the unfenced control plots were intended to detect possible enclosure artifacts.

The circular enclosures were made from white polyethylene and were 1.42 m in diameter and 1.2 m in height. They were pushed 20 to 30 cm into the sediment. Care was taken during the setup in spring 2004 not to damage the reed shoots. All individual enclosures were equipped with three or four outdoor aquarium heaters each (ProTemp 300 Watt; JBL, Neuhofen, Germany) to achieve or maintain the desired temperature. Aquarium pumps (universal hobby centrifugal pump; 300 liters h^{-1}; EHEIM GmbH & Co. KG, Deizisau, Germany) were connected to the heaters in all enclosures to achieve an even temperature distribution and maintain a weak but constant water circulation. Temperature was controlled by microprocessor-based temperature regulators (type FCR-13A; Roth and Co. AG, Oberuzwil, Switzerland).

Nitrogen was supplied every 4 weeks after exchanging one-third to half of the water in the enclosures, depending on the lake water level. To minimize changes in the ionic composition of the water, nitrate was added as $Ca(NO_3)_2$ dissolved in deionized H_2O. The amounts of nitrate added at each occasion were calculated based on nitrate concentrations in the lake measured in previous years at about the time of the addition. The target concentration was 5× the ambient concentration. From 10 November 2004 to 9 November 2005 the total added N load was 10.7 g m^{-2}.

Leaf litter preparation and processing. Fully brown leaf blades of *Phragmites australis* were collected from standing dead shoots near the shore at 120 to 150 cm above the lake water level. Leaves were collected in November 2004 and air dried. Weighed portions (5.0 ± 0.1 g) were enclosed in fine-mesh (0.5 by 0.5 mm) and coarse-mesh (9 by 9 mm) litter bags and submerged in enclosures and unfenced control plots in the marsh. One fine-mesh and one coarse-mesh bag were removed from each enclosure and control plot after 4.5 months (spring), when 26 to 77% of the initial litter dry mass was lost, and after 7.5 months (summer), when 47 to 91% of the litter dry mass was lost. A few empty litter bags collected at the second sampling date were excluded from the data analysis. The litter samples were placed in a cooler and returned to the laboratory, where the individual leaves were carefully cleaned with a jet of water to remove adhering debris and macroinvertebrates.

Subsamples from each litter bag were taken to determine bacterial community profiles, bacterial biomass, fungal biomass, and microbial respiration. For bacterial community profiles, one disc (8 mm in diameter) was cut from each of 12 leaves per litter bag and stored at −80°C for later analysis. To determine bacterial biomass, one leaf disc (11.8 mm in diameter) was cut from each of six leaves per litter bag and stored in 10 ml of formalin (2%, vol/vol) buffered with sodium pyrophosphate (0.1%, wt/vol). These samples were stored at 4°C. A total of five leaf discs (11.8 mm in diameter) per litter bag were frozen, lyophilized, and stored in 10 ml alkaline (0.8% KOH) methanol at −20°C until analyzed for ergosterol as a measure of fungal biomass. The dry mass of the lyophilized leaf discs was used to estimate the dry mass of the discs used for other analyses. Representative subsamples corresponding to 100 to 200 mg dry mass of the remaining leaf material were used to determine microbial respiration rates.

Bacterial community analysis. Genomic DNA was extracted from the litter samples with the MoBio Soil Ultra clean DNA extraction kit (MoBio, Laboratories, Inc., Carlsbad, CA). One aliquot of the extract was stored at −20°C and the other at 4°C to avoid repeated freeze-thaw cycles. The extracted DNA was amplified by PCR using standard primers annealing to the conserved regions of the 16S rRNA genes corresponding to positions 341 and 534 in *Escherichia coli.* The primer for position 341 contained an additional 40-nucleotide GC-rich sequence (GC clamp) at its 5′ end, which is necessary to separate PCR products by denaturing gradient gel electrophoresis (DGGE) (39). The primer sequences were 5′-GC-clamp-CC TAC GGG AGG CAG CAG-3′ for forward primer Eub341-GCf and 5′-ATT ACC GCG GCT GCT GG-3′ for reverse primer Eub534r, and the GC clamp sequence was 5′-CGC CCG CCG CGC GCG GCG GGC GGG GCG GGG GCA CGG GGG G-3′ (39).

PCR amplification was performed on a Touchgene gradient thermal cycler (Techne, Witec AG, Littau, Switzerland). For a reaction volume of 100 μl, we used 1 to 3 μl of DNA extract, 1× PCR buffer with $MgCl_2$ (final concentration, 1.5 mM) (MP Biomedicals, QBIOgene, Illkirch, France), 0.1 mg ml^{-1} bovine serum albumin (BSA), 0.2 mM each deoxynucleoside triphosphate (dNTP), 1 μM each primer, and 5 U of *Taq* polymerase (MP Biomedicals, QBIOgene, Illkirch, France). To reduce the nonspecific binding of primers to nontarget DNA, DNA was first denatured for 5 min at 95°C before adding *Taq* polymerase. Thirty-five cycles were performed with denaturation steps of 1 min at 95°C, annealing of primers for 1 min at 55°C, and elongation for 1 min at 72°C. The PCR program ended with a final extension of 5 min at 72°C.

A 10% acrylamide–bis-acrylamide gel (37.5:1; Sigma-Aldrich, Schnelldorf, Germany) with a denaturing gradient of 37.5 to 60.0% was poured within 3 to 4 min using a model 475 gradient former from Bio-Rad (Reinach, Switzerland). The resulting gel (16 by 16 cm) was 1 mm thick. The solution to prepare the gel was made of 1× TAE buffer (40 mM Tris base, 20 mM glacial acetic acid, 1 mM EDTA; pH 8) and the corresponding amounts of denaturant (100% denaturant corresponds to 7 M urea and 40% formamide, respectively). Gels were run for 10 min at 30 V, followed by 16 h at 65 V (total of 1,051 V · h) in a D-Code universal mutation detection system connected to a PowerPac universal power supply (Bio-Rad Life Science, Reinach, Switzerland). The running temperature was constant at 60°C. Bands were detected under UV light after staining the gel for 1 h with 1× concentrated GelStar (Cambrex Bio Science Inc., Rockland, ME) in 1× TAE. Images of the gels then were taken with a digital camera (Bio-Rad Gel Doc XR system) and analyzed using Bio-Rad Quantity One software version 4.6. Bands first were automatically marked and then checked individually for distinctness on the image. Only bands that were visible on the image by the naked eye were considered for matching.

Bacterial and fungal biomass. Numbers of bacteria were determined by epifluorescence microscopy after detaching and staining bacterial cells with SYBR green II (Invitrogen Molecular Probes, Eugene, OR) by following the protocol of Buesing (7). Samples were sonicated with an ultrasonic probe (Branson Sonifier 250; Branson Ultrasonics Corp., Danbury, CT) (10) and transferred onto an Anodisc filter (0.2-μm pore size, 25-mm diameter; Whatman, Maidstone, United Kingdom), and the bacterial cells were stained with SYBR green II and viewed at ×1,000 magnification under an Axioskop 2 epifluorescence microscope (excitation filter BP, 450 to 490 nm; beam splitter FT, 510 nm; emission filter LP, 520 nm; Carl Zeiss AG, Feldbach, Switzerland). Digital images were taken from 16 microscopic fields per filter with a cooled charge-coupled device (CCD) camera (Photometrics SenSys 0400; Roper Scientific, Trenton, NJ). Image analysis software (MetaMorph 4.6; Universal Imaging Corp., Downingtown, PA) was used to analyze at least 10 images or 400 cells per filter. Bacterial biovolumes (V, in μm^3) were determined by assuming that ovoid bacteria were rotational ellipsoids. All other morphotypes were considered cylinders with hemispherical ends. Bacterial carbon (in fg) was derived from the relationship $DM = 435 \times V^{0.86}$ (37), where DM is the cell dry mass, and bacterial carbon was assumed to be 50% of the cell dry mass (43).

Fungal biomass was determined by extracting and quantifying ergosterol (22). Ergosterol was extracted in 10 ml KOH-methanol (80°C for 30 min), and the extract was passed over solid-phase extraction (SPE) cartridges (24). Ergosterol in the eluate was purified by high-pressure liquid chromatography (HPLC). The HPLC was performed with a Jasco PU-980 liquid chromatography system (Tokyo, Japan) connected to a Jasco AS-950 autosampler, a LichroSpher 100 RP-18 column (0.46 by 25 cm) (Merck Inc., Darmstadt, Germany), and a Jasco MD 2010 Plus multiwavelength detector set at 282 nm. The column temperature was set at 33°C (Waters temperature control module; Waters AG, Baden-Dättwil, Switzerland). Twenty or 50 μl of the final sample extract was injected. The

retention time of ergosterol was 7.5 min with 100% HPLC-grade methanol at a flow rate of 1.5 ml min^{-1} (34). Ergosterol was quantified based on comparison to a commercial standard (>97% purity; Fluka, Sigma-Aldrich, Schnelldorf, Germany) and converted to fungal biomass and carbon, respectively, based on a fungal ergosterol content of 7.3 mg g^{-1} dry mass and 43% carbon in fungal dry mass (9).

Microbial respiration. Microbial respiration was used as an indicator of microbial activity. It was determined by measuring dissolved oxygen consumption over time. Measurements in spring were made 1 week after all other measurements and only for samples from blocks 1 and 2. In summer, respiration rates were determined for all samples. A litter subsample corresponding to 100 to 200 mg dry mass was placed in glass vials containing 30 ml of filtered (GF/F filter; Whatman, Maidstone, United Kingdom) lake water. Samples were acclimated to the *in situ* temperature before measuring the oxygen concentration at the *in situ* temperature in 30-s intervals for 30 min using YSI electrodes (model 5357; YSI Inc., Yellow Springs, OH). Oxygen consumption rates were corrected for the electrode drift, which was determined both before and after samples were measured. Drift usually was small. The litter used for respiration measurements was dried at 80°C for 3 days and weighed to the nearest 0.1 mg to calculate respiration rates per g of litter dry mass. The resulting rates were temperature corrected based on a Q_{10} of 2.5, which had been determined specifically for microbes on decomposing litter at the study site (A. Hammrich, personal communication). (Q_{10} refers to the multiplication factor by which respiration rates are increased when the temperature rises by 10°C.) The goal of the temperature normalization was to facilitate comparisons between sampling dates and to assess effects caused by long-term warming rather than immediate physiological responses to elevated temperature.

Statistics. Analysis of variance (ANOVA), with temperature and N as factors and the location in the reed stand as a blocking factor, was used to test for differences in bacterial biomass, fungal biomass, and microbial respiration. These analyses were performed with Systat 11 (Systat Software GmbH, Erkrath, Germany). Samples from the unfenced control plots were excluded from all ANOVAs but separately compared to samples from the control enclosures to reveal any enclosure artifacts (paired *t* tests). The bacterial community structure was analyzed by nonmetric multidimensional scaling (NMDS) using the freeware program PAST, which was downloaded from http://folk.uio.no/ohammer/past (27). The Dice coefficient was used to assess similarities between community profiles resulting from different experimental treatments (27). Three dimensions were included. The algorithm in PAST was run 11 times for each data set, and the outcomes with the lowest stress values are presented and used to interpret the data. To assess whether the groupings emerging from NMDS analyses were statistically significant, we used multiresponse permutation procedures (MRPP), a nonparametric analysis, as implemented in PC-ORD (see http://home.centurytel.net/~mjm/book.htm). The reported results refer to separate analyses comparing temperature and nitrogen treatments, respectively. Samples from one of the heated enclosures were excluded from the data analysis and presentations, since temperature in this enclosure was not well controlled during the experiment (see below).

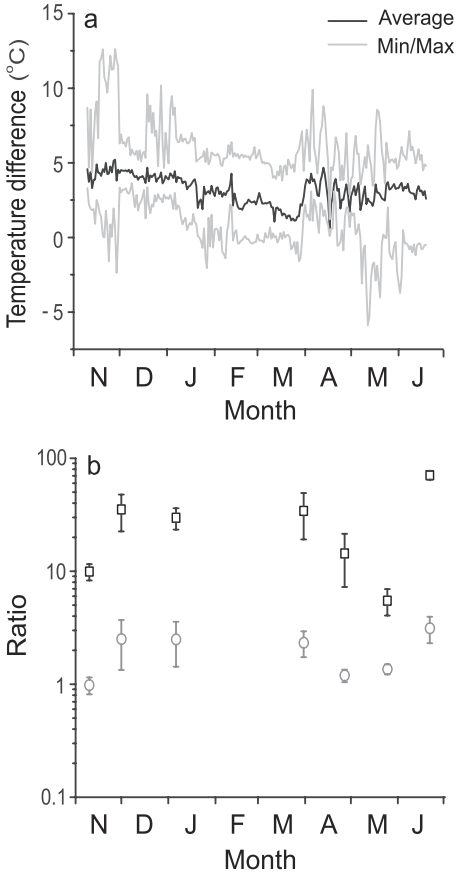

FIG. 1. (a) Average temperature difference between heated and unheated enclosures during a warming and nitrogen fertilization experiment in a freshwater marsh. (b) Ratios of NO_3^- concentrations of unfertilized (○) and fertilized (□) enclosures before and after water exchange and fertilization, respectively. Absolute background NO_3^- concentrations in the lake and unfertilized plots varied during the year from 20 to 3,410 μg N $liter^{-1}$.

RESULTS

Experimental conditions. During the experiment, heated enclosures were 3.2°C warmer on average than control enclosures and unfenced control plots (Fig. 1a), indicating that experimental warming during the experiment was successful overall. The temperature control of all enclosures was not always perfect, however. In particular, temperature differences between one heated enclosure and the corresponding control enclosure were below 1.5°C 55% of the time, below 1°C 40% of the time, and sometimes they were even negative.

Nitrate concentration averages during the period November 2004 to June 2005 ranged from 103 to 653 μg N $liter^{-1}$ in the unfertilized enclosures and unfenced control plots and from 245 to 2,699 μg N $liter^{-1}$ in fertilized enclosures. Enclosures always showed a strong increase in nitrate concentration after the experimental addition of nitrate (Fig. 1b). However, concentrations invariably dropped to background levels within a month or faster. Tracer experiments with fluorescein diacetate indicated that these declines in nitrate concentration were not due to dilution caused by hydrological exchanges between enclosures and the marsh (A. Hammrich, personal communication).

Microbial respiration. Temperature-corrected respiration rates did not significantly differ among treatments (Fig. 2). In spring, the rates were similar for leaves from coarse-mesh and fine-mesh bags, with an average of 0.20 ± 0.01 (means ± standard errors [SE]) mg O_2 g^{-1} dry mass h^{-1}. However, differences between mesh sizes were apparent in the summer ($P < 0.01$). Average respiration rates for fine-mesh bags were 25% lower in summer (0.15 ± 0.01 mg O_2 g^{-1} dry mass h^{-1}) than in spring ($P < 0.001$), whereas rates in coarse-mesh bags were similar at both times (0.193 ± 0.005 mg O_2 g^{-1} dry mass h^{-1}). This resulted in a significant difference between fine-mesh and coarse-mesh bags in summer ($P < 0.01$), with higher respiration rates associated with litter from coarse-mesh bags. The highest respiration rates were measured with litter from coarse-mesh bags from the unfenced control plots, where rates were significantly higher than those in control enclosures ($P = 0.01$).

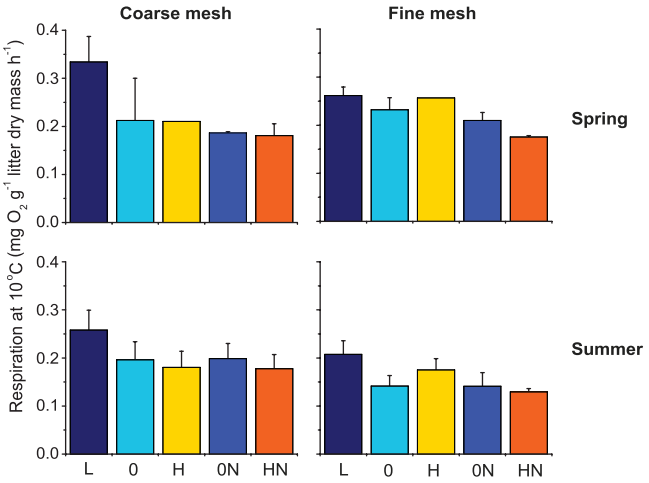

FIG. 2. Temperature-corrected rates of microbial respiration associated with decomposing leaf litter from coarse-mesh (left) and fine-mesh (right) bags collected in spring (upper half) and summer (lower half). L, unfenced lake control plot; 0, ambient temperature in enclosures; H, heated enclosures; 0N, ambient temperature and NO_3^- addition; HN, heated and NO_3^- addition. Error bars denote standard errors with $n = 2$ for spring and $n = 3$ to 4 for summer. No error bar indicates $n = 1$.

Bacterial community structure. The number of identified bands on DGGE gels obtained with samples from fine-mesh litter bags was 15 to 35% lower in heated enclosures ($P < 0.01$). No significant temperature effect emerged with samples from coarse-mesh bags, although the summer samples showed the same tendency as those from the fine-mesh bags (Fig. 3). Most strikingly, litter from coarse-mesh bags yielded band numbers 1.5 to more than 2 times higher than those from litter from fine-mesh bags. In summer, the number of bands from samples in coarse-mesh bags also was higher than that in spring ($P < 0.05$). The number of bands from the unfenced control plots in the marsh did not differ from that in the control enclosures.

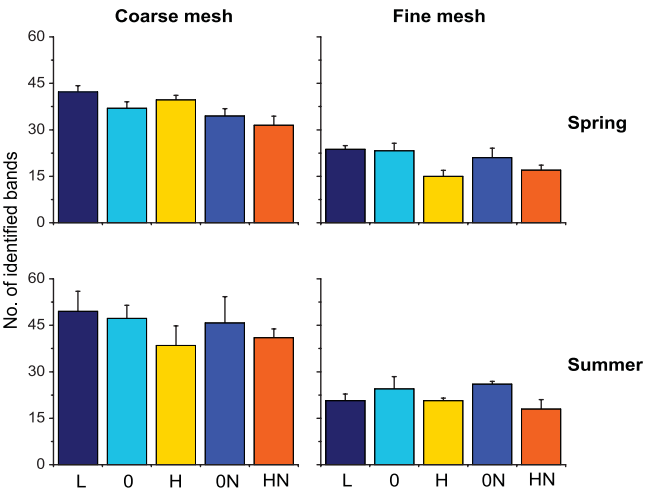

FIG. 3. Number of identified DGGE bands per litter sample from coarse-mesh (left) and fine-mesh (right) bags collected in spring (upper half) and summer (lower half). L, unfenced lake control plot; 0, ambient temperature in enclosures; H, heated enclosures; 0N, ambient temperature and NO_3^- addition; HN, heated and NO_3^- addition. Error bars denote standard errors, with $n = 2$ to 4.

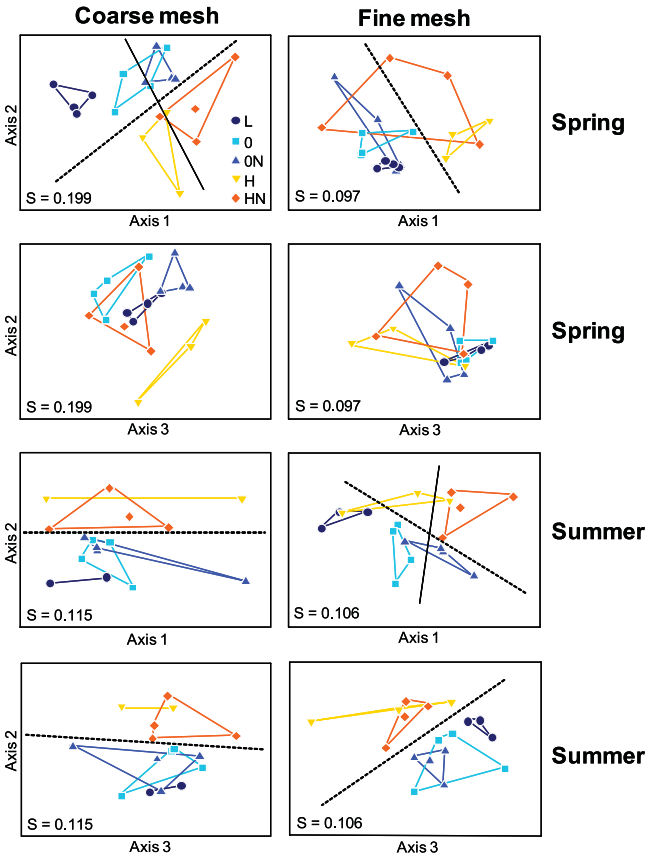

FIG. 4. Nonmetric multidimensional scaling (NMDS) projections on three axes showing the grouping of bacterial communities associated with decomposing leaf litter from coarse-mesh (left) and fine-mesh (right) bags collected in spring (upper half) and summer (lower half). Individual treatment groups are emphasized by color and convex hulls. Dashed lines separate communities exposed to ambient and elevated temperature, respectively, and the solid line divides communities from fertilized and unfertilized enclosures. S, stress; L, unfenced lake control plot; 0, ambient temperature in enclosures; H, heated enclosures; 0N, ambient temperature and NO_3^- addition; HN, heated and NO_3^- addition.

NMDS analysis revealed a clear grouping of bacterial communities experiencing either experimental warming or ambient temperature (Fig. 4). This grouping according to temperature is apparent on the projections of axes 1 and 2, of axes 2 and 3, or both, for both mesh sizes and both sampling dates. These NMDS results are supported by MRPP, which produced consistent significant differences between bacterial communities from heated and unheated enclosures for both sampling dates and mesh sizes (MRPP test statistic $A = 0.052$ to 0.112; $P = 0.014$ to < 0.001). Nitrate addition did not result in as clear-cut and consistent a grouping of bacterial communities as that of experimental warming (Fig. 4), although partial separations were evident, particularly for communities on litter from coarse-mesh bags collected in spring ($A = 0.040$; $P < 0.05$). A similar tendency also was apparent for bacterial communities from fine-mesh bags taken in summer ($A = 0.029$; $P = 0.080$). Bacterial communities on litter from unfenced control plots

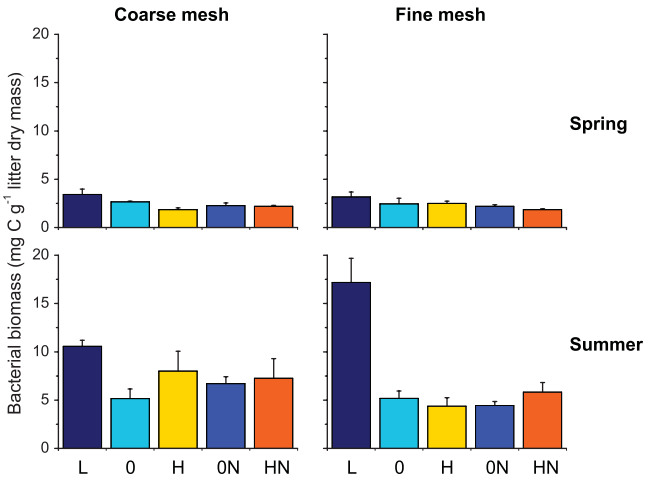

FIG. 5. Bacterial biomass associated with decomposing leaf litter from coarse-mesh (left) and fine-mesh (right) bags sampled in spring (upper half) and summer (lower half). L, unfenced lake control plot; 0, ambient temperature in enclosures; H, heated enclosures; 0N, ambient temperature and NO_3^- addition; HN, heated and NO_3^- addition. Error bars denote standard errors, with $n = 3$ to 4.

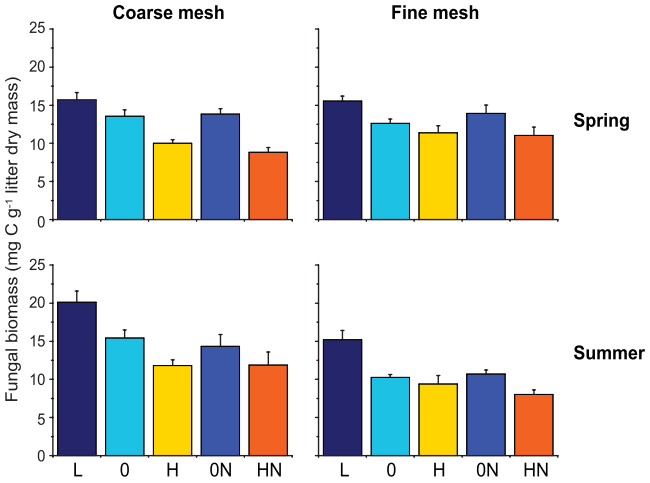

FIG. 6. Fungal biomass associated with decomposing leaf litter from coarse-mesh (left) and fine-mesh (right) bags collected in spring (upper half) and summer (lower half). L, unfenced lake control plot; 0, ambient temperature in enclosures; H, heated enclosures; 0N, ambient temperature and NO_3^- addition; HN, heated and NO_3^- addition. Error bars denote standard errors, with $n = 3$ to 4.

always clustered most closely together, indicating lower variability among control samples in the open marsh than among communities from replicate enclosures.

Microbial biomass. Bacterial biomass on decomposing litter ranged from 1.4 to 18.9 mg C g^{-1} litter dry mass, with an overall average of 2.25 ± 0.15 (SE) mg C g^{-1} litter dry mass (Fig. 5). Bacterial biomass generally was two times (fine-mesh bags) or three times (coarse-mesh bags) higher in summer than in spring ($P_{fine} < 0.001$; $P_{coarse} < 0.01$). It also differed significantly between litter from coarse-mesh and fine-mesh bags in summer ($P < 0.05$) but not in spring. Neither warming nor N addition had an effect on bacterial biomass at any time. Higher biomass was recorded on samples from the unfenced control plots in summer, particularly in fine-mesh bags, whereas differences between unfenced control plots and enclosures in spring were much smaller and not significant (Fig. 5).

Fungal biomass in decomposing litter was higher than bacterial biomass, ranging from 6.6 to 22.5 mg C g^{-1} litter dry mass (Fig. 6) with an overall average of 12.6 ± 2.0 (SE) mg C g^{-1} litter dry mass. Fungal biomass in fine-mesh and coarse-mesh bags was very similar in spring (~12 mg C g^{-1} litter dry mass). However, by summer, fungal biomass in litter from fine-mesh bags had decreased by 17% ($P < 0.01$), whereas it had increased in coarse-mesh bags by 19% ($P < 0.05$). Warming had a significant negative effect on fungal biomass in both seasons and for both mesh sizes ($P < 0.05$), whereas N addition produced no significant effect ($P > 0.40$). Litter from the unfenced control plots showed consistently higher fungal biomass than litter submerged in the control enclosures ($P < 0.05$).

DISCUSSION

Temperature effects. One of the clearest effects observed in our experiment was a shift in bacterial communities in response to simulated climate warming. Although published evidence on microbial responses to experimental warming under field conditions is scarce (4, 30), observational data suggest that microbial communities can be greatly influenced by temperature regimes. For example, decreasing bacterial diversity was found along a temperature gradient in an artificially aerated lagoon (50), and soil bacterial communities differed along a thermal gradient (35 to 65°C) resulting from recent geothermal activity in Yellowstone National Park, with apparently less complex communities in heated than in geothermally unaffected soils (44). Especially pronounced community shifts are thought to occur when microbes are exposed to temperatures not previously experienced (47). This idea was sparked by results of a transplantation experiment where soil bacterial communities changed when moved from a shaded site (maximum temperature, 24°C) to an open site with temperatures of up to 33°C, whereas the reciprocal transfer from the open to the shaded site did not induce a community change (47). The asymmetric response was attributed to the fact that 24°C, but not 33°C, was within the temperature range experienced by the transplanted community before the soil was moved. Finlay et al. (16) suggested, more generally, that changes in microbial communities often occur when environmental factors vary with high amplitudes or frequencies. The geothermal gradient and soil transplantation studies (44, 47) lend support to this notion. However, warming in our experiment produced a similar bacterial community shift, with apparent community complexity declining at least in fine-mesh bags. These changes occurred even though the temperature rise we imposed was minimal compared to the geothermal heating in Yellowstone National Park, and it also was well within the range normally experienced at the site. Clearly, therefore, unprecedented or large amplitudes are not a compulsory condition for temperature effects on bacterial community structure to play out. This suggests that even moderate climate warming can have a bearing on bacterial communities associated with decomposing litter in freshwater marshes.

If shifts in microbial communities occur under climate change, it is important to understand whether they are accompanied by changes in microbial growth and activity or whether functional redundancies in diverse communities buffer against such consequences (5, 49). That total bacterial biomass was unaffected by simulated climate warming in spite of changes in community structure indicates that some bacteria could indeed compensate for the decline of others, lending support to the redundancy hypothesis, which might better be referred to as the degeneracy hypothesis (15, 17). This conclusion is corroborated by the lack of warming effects on temperature-normalized microbial respiration associated with the decomposing litter, which was remarkably similar among treatments.

The lower fungal biomass observed in our elevated-temperature treatments indicates that fungi respond more sensitively to climate warming than bacteria. Fungi in both salt and freshwater marshes accumulate substantial biomass on leaves during the decomposition of standing dead shoots (20, 36, 40, 41), but fungal biomass declines when the leaves eventually are dropped (35, 36, 46). This decline supposedly reflects stressful conditions when the fungi associated with the leaves move from the plant canopy to the sediment surface (36, 46). Experimental warming could have exacerbated this stress. It is not clear whether the greater fungal than bacterial sensitivity to warming observed in our experiment is due to a direct temperature influence or to indirect mechanisms, such as more intense grazing on fungi in warmer water. However, the greater reduction of fungal biomass in coarse-mesh litter bags suggests that indirect effects play a role.

Why did the lower fungal biomass in heated enclosures not result in reduced temperature-normalized microbial respiration? A possible explanation is that fungi were relatively inactive at the time when samples were taken 4.5 and 7.5 months after the submergence of the leaf litter in enclosures. This interpretation is in accordance with (i) the relatively low fungal biomass at the time of sampling (for comparison, see reference 21), (ii) declines in fungal biomass observed after the detachment of leaves from emergent macrophyte shoots (as described above), and (iii) the finding that bacterial productivity on submerged plant litter in freshwater marshes appears to assume great importance (9, 45). Thus, it is conceivable that at the time of sampling in spring and summer, when leaf litter was in an advanced stage of submerged decomposition, fungi contributed relatively little to the overall activity of the litter-associated microbial communities.

Nitrogen effects. Nitrogen enrichment had only a small effect on bacterial community structure, since the significant separation of unfertilized and fertilized communities was evident only in samples taken in spring from coarse-mesh litter bags. Likewise, the lack of N effects on apparent bacterial species richness (i.e., DGGE band numbers), bacterial biomass, fungal biomass, and microbial respiration suggests that the microbes associated with decomposing leaf litter in the marsh were largely unaffected by nitrogen enrichment. This unresponsiveness could be due to a legacy of anthropogenic N inputs to ecosystems in central Europe in general (18), in the sense that even the communities present in the control treatments might have been adapted to chronically high nitrogen supplies. Dissolved nitrate concentrations in the marsh water were indeed high during winter, and high N/P ratios indicated that P rather than N usually was the element in shortest supply during our experiment, even when no N was added. Similarly, unresponsive communities analyzed by DGGE have been found in sediments of salt marshes receiving experimental nutrient additions (both N and P) of 15-fold above ambient levels (6). Conversely, shifts in soil microbial communities have been found in experimentally fertilized grasslands (30) and forests (2, 13), and even in fertilized agricultural fields (32). Therefore, caution is needed in order not to discard anthropogenic N deposition prematurely as a factor of global change affecting microbial communities.

Temporal changes and mesh size effects. A lack of clear indication for pronounced changes in apparent bacterial richness between spring and summer in our experiment is consistent with the weak seasonal patterns in bacterial communities found in the same marsh on naturally deposited plant litter (8). Similar information from other freshwater marshes is lacking, but in a salt marsh (6) and other aquatic environments (1, 3, 12, 33), temporal changes of bacterial communities have been found commonly. Moreover, consistently large increases in bacterial biomass between spring and summer, which in fine-mesh bags were accompanied by decreases in fungal biomass, indicate that even in our study important temporal shifts occurred in the litter-associated communities. This suggests that microbial succession on decomposing plant litter in marshes is not always associated with detectable changes in apparent species richness (as assessed from DDGE bands) and that complementary microbial parameters, such as total bacterial and fungal biomass, can provide added insights.

In contrast to the limited differences between sampling dates in spring and summer, there were clear responses of the microbes to differences in habitat conditions between coarse-mesh and fine-mesh litter bags. This was reflected primarily by a greatly reduced number of identified bacterial DGGE bands in samples from fine-mesh bags, accompanied in summer by reduced fungal biomass and temperature-normalized respiration rates. These discrepancies between coarse-mesh and fine-mesh litter bags imply an influence of dissolved oxygen concentration or other factors related to water exchange, since by summer benthic algae had overgrown the fine-mesh bags and might have hampered water circulation inside. However, we found no indications of anoxic conditions in the litter bags. Alternatively, differences in the microbial communities associated with litter in fine-mesh and coarse-mesh bags could involve detritivorous invertebrates. Selective feeding by isopods (*Asellus aquaticus* L.) and detritivorous caddis-flies (mainly Limnephilidae) could directly influence the microbial decomposers and affect microbial habitat conditions through changes of litter quality. Irrespective of the relative importance of these possible causes, the striking difference in DGGE band numbers observed between coarse-mesh and fine-mesh litter bags in our experiment clearly points to considerable potential for at least bacterial community shifts in response to changing environmental conditions, altered biotic interactions, or both.

ACKNOWLEDGMENTS

We thank Arne Hammrich, Daniel Steiner, Doris Hohmann, Sabina Käppeli, and Markus Schindler for assistance during field and laboratory work; Helmut Bürgmann and Francisco Vazquez for support

during DGGE analyses; Richard Pearson for performing the MRPP analyses; and Arne Hammrich for providing unpublished data.

This study was funded by the Swiss National Science Foundation (SNF; grant no. 3100A0-108441) and the Swiss State Secretariat for Education and Research (SER) through the Euro-limpacs project supported under the 7th Framework Programme of the EU Commission.

REFERENCES

1. **Allgaier, M., and H. P. Grossart.** 2006. Seasonal dynamics and phylogenetic diversity of free-living and particle-associated bacterial communities in four lakes in northeastern Germany. Aquat. Microb. Ecol. **45:**115–128.
2. **Allison, S. D., C. A. Hanson, and K. K. Treseder.** 2007. Nitrogen fertilization reduces diversity and alters community structure of active fungi in boreal ecosystems. Soil Biol. Biochem. **39:**1878–1887.
3. **Andersson, A. F., L. Riemann, and S. Bertilsson.** 2010. Pyrosequencing reveals contrasting seasonal dynamics of taxa within Baltic Sea bacterioplankton communities. ISME J. **4:**171–181.
4. **Barnard, R., et al.** 2006. Several components of global change alter nitrifying and denitrifying activities in an annual grassland. Funct. Ecol. **20:**557–564.
5. **Bell, T., et al.** 2009. Microbial biodiversity and ecosystem functioning under controlled conditions and in the wild, p. 121–133. *In* S. Naeem, D. E. Bunker, A. Hector, M. Loreau, and C. Perrings (ed.), Biodiversity, ecosystem functioning, and human wellbeing: an ecological and economic perspective. Oxford University Press, Oxford, United Kingdom.
6. **Bowen, J. L., B. C. Crump, L. A. Deegan, and J. E. Hobbie.** 2009. Salt marsh sediment bacteria: their distribution and response to external nutrient inputs. ISME J. **3:**924–934.
7. **Buesing, N.** 2005. Bacterial counts and biomass determination by epifluorescence microscopy, p. 203–208. *In* M. A. S. Graça, F. Bärlocher, and M. O. Gessner (ed.), Methods to study litter decomposition: a practical guide. Springer, Dordrecht, Netherlands.
8. **Buesing, N., M. Filippini, H. Bürgmann, and M. O. Gessner.** 2009. Microbial communities in contrasting freshwater marsh microhabitats. FEMS Microbiol. Ecol. **69:**84–97.
9. **Buesing, N., and M. O. Gessner.** 2006. Benthic bacterial and fungal productivity and carbon turnover in a freshwater marsh. Appl. Environ. Microbiol. **72:**596–605.
10. **Buesing, N., and M. O. Gessner.** 2002. Comparison of detachment procedures for direct counts of bacteria associated with sediment particles, plant litter and epiphytic biofilms. Aquat. Microb. Ecol. **27:**29–36.
11. **Cebrian, J., and J. Lartigue.** 2004. Patterns of herbivory and decomposition in aquatic and terrestrial ecosystems. Ecol. Monogr. **74:**237–259.
12. **Crump, B. C., G. W. Kling, M. Bahr, and J. E. Hobbie.** 2003. Bacterioplankton community shifts in an arctic lake correlate with seasonal changes in organic matter source. Appl. Environ. Microbiol. **69:**2253–2268.
13. **Demoling, F., L. O. Nilsson, and E. Bååth.** 2008. Bacterial and fungal response to nitrogen fertilization in three coniferous forest soils. Soil Biol. Biochem. **40:**370–379.
14. **Dilly, O., J. Bloem, A. Vos, and J. C. Munch.** 2004. Bacterial diversity in agricultural soils during litter decomposition. Appl. Environ. Microbiol. **70:**468–474.
15. **Edelman, G. M., and J. A. Gally.** 2001. Degeneracy and complexity in biological systems. Proc. Natl. Acad. Sci. U. S. A. **98:**13763–13768.
16. **Finlay, B. J., S. C. Maberly, and J. I. Cooper.** 1997. Microbial diversity and ecosystem function. Oikos **80:**209–213.
17. **Franklin, R. B., J. L. Garland, C. H. Bolster, and A. L. Mills.** 2001. Impact of dilution on microbial community structure and functional potential: comparison of numerical simulations and batch culture experiments. Appl. Environ. Microbiol. **67:**702–712.
18. **Galloway, J. N., et al.** 2004. Nitrogen cycles: past, present, and future. Biogeochemistry **70:**153–226.
19. **Galloway, J. N., et al.** 2008. Transformation of the nitrogen cycle: recent trends, questions, and potential solutions. Science **320:**889–892.
20. **Gessner, M. O.** 2001. Mass loss, fungal colonization and nutrient dynamics of *Phragmites australis* leaves during senescence and early aerial decay. Aquat. Bot. **69:**325–339.
21. **Gessner, M. O., V. Gulis, K. A. Kuehn, E. Chauvet, and K. Suberkropp.** 2007. Fungal decomposers of plant litter in aquatic ecosystems, p. 301–324. *In* C. P. Kubicek and I. S. Druzhinina (ed.), Microbial and environmental relationships, 2nd ed., vol. 4. Springer-Verlag, Berlin, Germany.
22. **Gessner, M. O., and S. Y. Newell.** 2002. Biomass, growth rate, and production of filamentous fungi in plant litter, p. 390–408. *In* C. J. Hurst, R. L. Crawford, G. R. Knudsen, M. J. McInerney, L. D. Stetzenbach, and M. Walter (ed.), Manual of environmental microbiology, 2nd ed. ASM Press, Washington, DC.
23. **Gessner, M. O., B. Schieferstein, U. Müller, S. Barkmann, and U. A. Lenfers.** 1996. A partial budget of primary organic carbon flows in the littoral zone of a hardwater lake. Aquat. Bot. **55:**93–105.
24. **Gessner, M. O., and A. L. Schmitt.** 1996. Use of solid-phase extraction to determine ergosterol concentrations in plant tissue colonized by fungi. Appl. Environ. Microbiol. **62:**415–419.
25. **Gessner, M. O., et al.** 2010. Diversity meets decomposition. Trends Ecol. Evol. **25:**372–380.
26. **Graça, M. A. S., F. Bärlocher, and M. O. Gessner (ed.).** 2005. Methods to study litter decomposition: a practical guide. Springer, Dordrecht, Netherlands.
27. **Hammer, O., D. A. T. Harper, and P. D. Ryan.** 2001. PAST: palaeontological statistics software package for education and data analysis. Palaeontol. Electron. **4:**9.
28. **Hobbs, R. J., E. Higgs, and J. A. Harris.** 2009. Novel ecosystems: implications for conservation and restoration. Trends Ecol. Evol. **24:**599–605.
29. **Hocking, P. J.** 1989. Seasonal dynamics of production, and nutrient accumulation and cycling by *Phragmites australis* (Cav.) Trin. ex Steudel in a nutrient-enriched swamp in inland Australia. 1. Whole plants. Austr. J. Mar. Freshwater Res. **40:**421–444.
30. **Horz, H. P., A. Barbrook, C. B. Field, and B. J. M. Bohannan.** 2004. Ammonia-oxidizing bacteria respond to multifactorial global change. Proc. Natl. Acad. Sci. U. S. A. **101:**15136–15141.
31. **IPCC.** 2007. Climate change 2007: the physical science basis. Contribution of Working Group I to the fourth assessment report of the Intergovernmental Panel on Climate Change. Cambridge University Press, Cambridge, United Kingdom.
32. **Iyyemperumal, K., and W. Shi.** 2007. Soil microbial community composition and structure: residual effects of contrasting N fertilization of swine lagoon effluent versus ammonium nitrate. Plant Soil **292:**233–242.
33. **Kent, A. D., et al.** 2004. Annual patterns in bacterioplankton community variability in a humic lake. Microb. Ecol. **48:**550–560.
34. **Komínková, D., K. A. Kuehn, N. Büsing, D. Steiner, and M. O. Gessner.** 2000. Microbial biomass, growth, and respiration associated with submerged litter of *Phragmites australis* decomposing in a littoral reed stand of a large lake. Aquat. Microb. Ecol. **22:**271–282.
35. **Kuehn, K. A., M. J. Lemke, K. Suberkropp, and R. G. Wetzel.** 2000. Microbial biomass and production associated with decaying leaf litter of the emergent macrophyte *Juncus effusus*. Limnol. Oceanogr. **45:**862–870.
36. **Kuehn, K. A., D. Steiner, and M. O. Gessner.** 2004. Diel mineralization patterns of standing-dead plant litter: implications for CO_2 flux from temperate wetlands. Ecology **85:**2504–2518.
37. **Loferer-Krößbacher, M., J. Klima, and R. Psenner.** 1998. Determination of bacterial cell dry mass by transmission electron microscopy and densitometric image analysis. Appl. Environ. Microbiol. **64:**688–694.
38. **Moore, J. C., et al.** 2004. Detritus, trophic dynamics and biodiversity. Ecol. Lett. **7:**584–600.
39. **Muyzer, G., E. C. Dewaal, and A. G. Uitterlinden.** 1993. Profiling of complex microbial-populations by denaturing gradient gel-electrophoresis analysis of polymerase chain reaction-amplified genes-coding for 16S ribosomal-RNA. Appl. Environ. Microbiol. **59:**695–700.
40. **Newell, S. Y.** 2001. Multiyear patterns of fungal biomass dynamics and productivity within naturally decaying smooth cordgrass shoots. Limnol. Oceanogr. **46:**573–583.
41. **Newell, S. Y., L. K. Blum, R. E. Crawford, T. Dai, and M. Dionne.** 2000. Autumnal biomass and potential productivity of salt marsh fungi from 29° to 43° north latitude along the United States Atlantic Coast. Appl. Environ. Microbiol. **66:**180–185.
42. **Nocker, A., M. Burr, and A. K. Camper.** 2007. Genotypic microbial community profiling: a critical technical review. Microb. Ecol. **54:**276–289.
43. **Norland, S.** 1993. The relationship between biomass and volume of bacteria, p. 303–307. *In* P. F. Kemp, B. F. Sherr, E. B. Sherr, and J. J. Cole (ed.), Handbook of methods in aquatic microbial ecology. Lewis Publishers, Boca Raton, FL.
44. **Norris, T. B., J. M. Wraith, R. W. Castenholz, and T. R. McDermott.** 2002. Soil microbial community structure across a thermal gradient following a geothermal heating event. Appl. Environ. Microbiol. **68:**6300–6309.
45. **Su, R., R. N. Lohner, K. A. Kuehn, R. Sinsabaugh, and R. K. Neely.** 2007. Microbial dynamics associated with decomposing *Typha angustifolia* litter in two contrasting Lake Erie coastal wetlands. Aquat. Microb. Ecol. **46:**295–307.
46. **Van Ryckegem, G., M. O. Gessner, and A. Verbeken.** 2007. Fungi on leaf blades of *Phragmites australis* in a brackish tidal marsh: diversity, succession, and leaf decomposition. Microb. Ecol. **53:**600–611.
47. **Waldrop, M. P., and M. K. Firestone.** 2006. Response of microbial community composition and function to soil climate change. Microb. Ecol. **52:**716–724.
48. **Webster, J. R., and E. F. Benfield.** 1986. Vascular plant breakdown in fresh-water ecosystems. Annu. Rev. Ecol. Syst. **17:**567–594.
49. **Wertz, S., et al.** 2006. Maintenance of soil functioning following erosion of microbial diversity. Environ. Microbiol. **8:**2162–2169.
50. **Yu, Z. T., and W. W. Mohn.** 2001. Bacterial diversity and community structure in an aerated lagoon revealed by ribosomal intergenic spacer analyses and 16S ribosomal DNA sequencing. Appl. Environ. Microbiol. **67:**1565–1574.