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## Invertebrate footprints on detritus processing, bacterial community structure, and spatiotemporal redox profiles

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**Abstract.** Detritus processing is driven by a complex interplay between macroinvertebrate and microbial activities. Bioturbation/feeding activities of invertebrates in sediments are known to influence decomposition rates. However, direct effects of invertebrates on bacterial communities and detritus processing remain ill-defined, mainly because identifying interactions between invertebrates and sediments is methodologically challenging. We incubated 5 macroinvertebrate species with various bioturbation/feeding traits separately in sediment-filled microcosms inoculated with bacterial communities for 5 d. At the end of the experiment, we assessed: 1) detritus processing (mass loss on ignition [LOI] and dissolved organic C accumulation in the overlying water [absorbance at 280 nm]), 2) bacterial community structure (intergenic spacer analysis [RISA]) and bacterial activity (electron transport system activity [ETSA]), and 3) development of redox potential (Eh) over time (with permanently installed microelectrodes). Invertebrates enhanced bacterial activity and detritus processing, and the magnitude depended on bioturbation/feeding traits. Bacterial community structure differed significantly between microcosms with burrowing invertebrates and microcosms with sediment-dwelling invertebrates. Eh profiles were similar among microcosms with invertebrates with similar bioturbation/feeding traits, but differed among microcosms with invertebrates with dissimilar bioturbation/feeding traits. Our results suggest that bioturbation by aquatic invertebrates mediates detritus processing, Eh dynamics, and structure of the microbial community. These findings highlight the significance of bioturbation and show the utility of spatiotemporal Eh dynamics as footprints reflecting functioning of benthic detrital food webs.

**Key words:** decomposition, aquatic invertebrates, bioturbation, functional traits, bacterial community structure, redox potential.

Decomposition and sequestration of organic C are central processes in ecosystem functioning (Odum and de la Cruz 1963, Carpenter 1980, Gessner et al. 2010). Organic matter processing is driven by a complex interplay between macroinvertebrate and

microbial activities, which act interdependently and in a facilitative manner. Interest in the effect of bioturbation on organic matter processing is high (Mermillod-Blondin and Rosenberg 2006, Nogaro et al. 2009) because up to 50% of the particulate organic matter (POM) pool becomes trapped in subsurface sediments (Herbst 1980, Metzler and Smock 1990). Macroinvertebrates alter sediments by modifying texture, distributing solid particles, and introducing O<sub>2</sub> into otherwise anoxic zones (Covich et al. 2004, Nogaro et al. 2009, Navel et al. 2010). Thus, biological and geochemical components of subsurface sediments might be coupled. However, direct effects of invertebrate bioturbation/feeding behavior on bacterial

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community structure remain ill-defined, especially in freshwater sediments.

Detritivorous invertebrates display a wide variety of bioturbation/feeding traits that differentially affect sediment biogeochemistry, bacterial activity, and detritus processing (Mermillod-Blondin et al. 2002, Jonsson and Malmqvist 2003, Nogaro et al. 2009). Invertebrates can be categorized by the way they affect the physicochemical and biogeochemical properties of the sediment (François et al. 1997, Gérino et al. 2003, Nogaro et al. 2009). Invertebrates may act as: 1) biodiffusers whose surface activity results in random downward mixing; 2) upward conveyors and 3) downward conveyors, whose activities move sediment vertically upward or downward, respectively; 4) regenerators that create open burrows that fill with surface particles when abandoned; and 5) gallery diffusors that create galleries of actively irrigated burrows. Identifying trait-specific signatures of sediment reworking would greatly enhance our capabilities to explain and predict ecosystem responses to changes in environmental pressure and declines in diversity, but identifying these interactions is methodologically challenging and laborious (Solan et al. 2004, Naeem and Bunker 2009, Birchenough et al. 2012). Spatial and temporal redox potential (Eh) profiles have been used to reflect biogeochemical processes and functioning of sediments in response to bioturbation (Hunting and van der Geest 2011, Vorenhout et al. 2011) and might provide useful biogeochemical signatures of species-specific bioturbation/feeding activities.

Our objectives were to evaluate whether bioturbation/feeding traits of aquatic invertebrate species differentially affect detritus processing and benthic microbial community structure and to test the utility of Eh profiles as biogeochemical signatures of types of bioturbation in laboratory microcosms.

## Methods

### *Microcosms and test organisms*

*Microcosms.*—We tested the effects of bioturbation/feeding activity on bacterial community structure, detritus processing, and redox geochemistry in the immediate environment of the invertebrates in laboratory microcosms. We constructed microcosms from sterilized 50-mL glass vials (25-mm diameter) filled with fine-grained, ignited quartz sand as mineral substrate (12.5 g, grain size: 0.1–0.5 mm, total sediment depth: 18 mm). We assumed that the size of the microcosms did not affect invertebrate behavior. We used 8 mg of freeze-dried, ground, and sieved stinging nettle (*Urtica dioica* L., <500 µm particle size)

as detritus. This plant often dominates the riparian zone of aquatic systems (Stief 2007). We filled microcosms with 35 mL of Dutch Standard Water (DSW; a standardized synthetic analog of common Dutch surface waters). DSW contains 200 mg CaCl<sub>2</sub>·2H<sub>2</sub>O, 180 mg MgSO<sub>4</sub>·7H<sub>2</sub>O, 100 mg NaHCO<sub>3</sub>, and 20 mg KHCO<sub>3</sub>/L demineralized H<sub>2</sub>O (pH 8.1, hardness 210 mg/L CaCO<sub>3</sub>, alkalinity 1.2 meq/L). We gently aerated the overlying water through needles without disturbing the sediment.

*Treatments and controls.*—We compared effects of 5 invertebrate species in microcosms with and without bacterial inocula on organic matter processing, bacterial community structure and activity, and sediment Eh profiles. Each treatment and control was replicated 7 times (140 microcosms). We inoculated half of the microcosms with a bacterial consortium (see below). We added individuals of a single invertebrate species (see below) to half of the microcosms (7 microcosms/species) with a bacterial consortium (invertebrate treatment) and to half of the sterile microcosms (control for contribution of invertebrate-derived bacteria). Microcosms inoculated with a bacterial consortium and no invertebrates were negative controls for the invertebrate treatments. We used sterile microcosms without invertebrates to calculate organic matter content at the initial time point in calculations of decomposition rate.

*Bacteria.*—We used bacterial communities from metabolically and taxonomically distinct strains of bacteria isolated from aquatic sediments. We assembled communities from overnight cultures of *Azospirillum brasiliense*, *Bacillus subtilis*, *Paenibacillus polymyxa*, *Pseudomonas putida*, *Sphingomonas paucimobilis*, *Micrococcus luteus*, *Streptomyces antibiotica*, *Pseudomonas stutzeri*, *Flavobacterium* sp., *Aeromonas salmonida*, *Paracoccus pantotrophus*, and *Aminobacter aminovorans* (obtained from the Fungal Biodiversity Centre, CBS-KNAW, Utrecht, The Netherlands), all grown in brain–heart broth (Merck, Darmstadt, Germany) and peptonized milk nutrient (Sigma–Aldrich, St. Louis, Missouri) (ratio 100:15). We standardized bacterial biomass for each strain by dilution to obtain an optical density at 600 nm (OD<sub>600</sub>) of 0.2. We inoculated each microcosm (except for the sterile microcosms) with 1 mL of bacterial suspension.

*Invertebrates.*—We selected aquatic invertebrates based on consensus regarding their bioturbation traits in the primary literature, availability, and performance under culture and laboratory conditions. We used 5 invertebrate species that represented 3 types of bioturbators as outlined by Usseglio-Polatera et al. (2000) and Nogaro et al. (2009). The isopod *Asellus aquaticus* and the amphipod *Gammarus pulex* are omnivorous sediment dwellers that act as biodiffusers, i.e., grazing the upper layer of detritus and

biofilms on sediment particles. Larvae of the non-biting midge *Chironomus riparius* create ventilated U-shaped tubes, feed on surface sediment material, and are considered gallery diffusors. The oligochaetes *Tubifex* spp. and *Lumbriculus variegatus* are both upward conveyors, i.e., deposit feeders that create burrowing networks in the sediment and defecate on the sediment surface.

#### *Experimental procedure*

At the start of the experiment, we inoculated microcosms with bacterial communities and left them undisturbed to allow stratification of the sediment, succession of the bacterial community, and partial degradation of detritus. We added invertebrates to the appropriate microcosms ( $n = 7$  microcosms per species) 40 h after bacterial inoculation. To standardize invertebrate biomass, we used relationships between length (for *C. riparius*, *A. aquaticus*, and *G. pulex*) or fresh mass (*Tubifex* spp. and *L. variegatus*) and dry mass (DM) based on 12 to 30 individuals/species. We added equal initial DM of invertebrates to the microcosms (mean  $\pm$  SD,  $0.35 \pm 0.03$  mg DM/microcosm). We used 1 small individual of *A. aquaticus* and *G. pulex* (5–7 mm in length) per microcosm, and 3 or 4 individuals of *C. riparius*, *Tubifex* spp., and *L. variegatus*/microcosm. After 5 d, we evaluated the influence of the invertebrate species on detritus processing, bacterial activity, bacterial community structure, and development of Eh.

#### *Organic matter processing*

We characterized detritus processing as sediment mass loss on ignition (LOI) and increase in DOC in the overlying water at the end of the experiment. After we collected 2 mL of sediment from each microcosm for measurement of bacterial activity and community structure (see below), we oven dried the remaining sediment and combusted it at 550°C for 24 h. We calculated LOI as mass loss relative to mass of sediments in sterile controls that did not contain invertebrates. We measured DOC as absorbance at 280 nm in the overlying water. We tested the data for normality (Lilliefors) and used 1-way analysis of variance (ANOVA) and Tukey's Honestly Significant Difference (HSD) post hoc test (Matlab version 7.2, Mathworks, Boston, Massachusetts) to identify differences among invertebrate treatments in inoculated microcosms, including inoculated controls with no invertebrates.

#### *Bacterial activity and community structure*

We assessed bacterial activity in the sediment by measuring electron transport system activity (ETSA)

following reduction of 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) to formazan (INTF) sensu Smith and McFeters (1997). In brief, we collected 1 mL of sediment, vortexed it with 1 mL of overlying medium, and centrifuged (short spin) it to deposit coarse material. We assayed the supernatants (400  $\mu$ L porewater with suspended bacteria) for ETSA with procedures recently described by Hunting et al. (2010). We used the same statistical analysis described above.

We collected sediment for deoxyribonucleic acid (DNA) analysis with 1 mL pipettes. We centrifuged the samples, air-dried them, and stored them at  $-20^{\circ}\text{C}$  until analysis. We extracted bacterial DNA from 2 g of sediment using PowerSoil extraction kits (Mo-Bio, Carlsbad, New Mexico). We assayed bacterial community structure in 3 replicates of each inoculated and sterile invertebrate treatment and 2 inoculated microcosms with no invertebrates with ribosomal intergenic spacer analysis (RISA) with universal 16–23S bacterial primers and polymerase chain reaction (PCR) amplification as described by Danovaro et al. (2006). We separated PCR-amplified fragments on 3.5% polyacrylamide gels and stained the gels with ethidium bromide. We analyzed RISA banding patterns with a Jaccard-based cluster analysis and 1-way analysis of similarity (ANOSIM), followed by a permutation based, Bonferroni-corrected pairwise comparison using PAST (Hammer et al. 2001).

#### *Redox potential profiles*

We visualized effects of invertebrate species on sediment geochemistry as vertical profiles of Eh recorded over time. We measured Eh in 3 replicates of each invertebrate treatment in inoculated microcosms, including inoculated controls with no invertebrates. We recorded Eh with permanently installed redox microelectrodes and a calomel reference electrode connected to a Hypnos data logger (MVH Consult, Leiden, The Netherlands), both of which are newly developed in our laboratory (Vorenhout et al. 2011). We constructed Eh microelectrodes from Au-plated printed circuit board and placed them permanently in the middle of the sediment cores to allow high-resolution measurement of Eh in subsurface sediments (each mm [0–7-mm] depth, 2-mm width, every 15 min) throughout the experiment. During the preincubation (first 40 h), we monitored Eh values and repositioned electrodes to ensure similarity in positioning with respect to the sediment surface among replicates. We converted Eh values to standard H-electrode output by adding 245 mV and generated contour charts with linear interpolation (DeltaGraph,

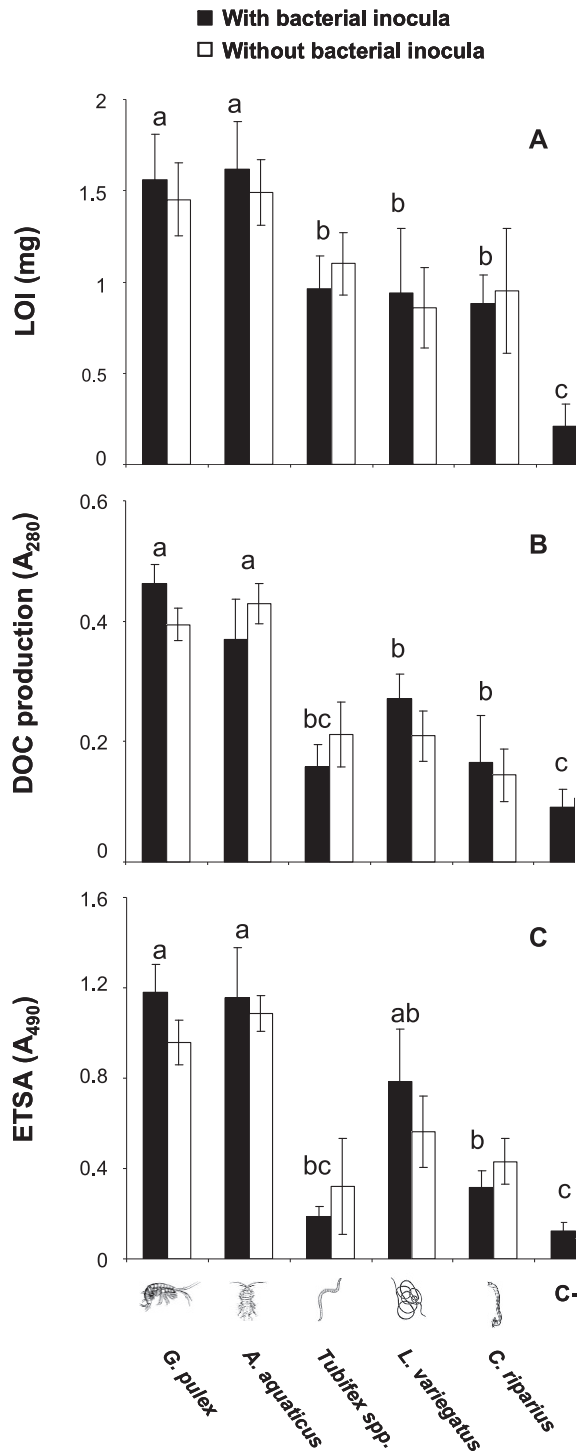


FIG. 1. Mean ( $\pm 1$  SD) loss of particulate organic matter on ignition (LOI) (A), dissolved organic C (DOC) in the overlying water column (absorbance [A] at 280 nm) (B), and bacterial electron transfer system activity (ETSA) (C) in *Gammarus pulex*, *Asellus aquaticus*, *Tubifex* spp., *Lumbriculus variegatus*, and *Chironomus riparius* treatments in microcosms with bacterial inocula, without bacterial inocula, and in controls (inoculated microcosms without invertebrates).

version 5.0; Red Rock Software, Salt Lake City, Utah). We used a general linear model (GLM) approach to analysis of covariance (ANCOVA) to compare measurements of Eh among invertebrate treatments (Engqvist 2005). The dependent variable was mean Eh in time at each depth, and depth was the covariate. In the ANCOVA, invertebrate treatments did not differ in slopes ( $p = 0.14$ ), a result that could have indicated depth dependence. Therefore, we removed the interaction term to test for the effect of invertebrate treatment with a Tukey's HSD post hoc test.

## Results

Bacterial activity and detritus processing did not differ between inoculated and sterile microcosms containing invertebrates for any invertebrate species (unpaired  $t$ -tests,  $p > 0.35$ ; Fig. 1A–C), a result suggesting that invertebrates contributed strongly to bacterial activity and detritus processing. Bacterial activity and detritus processing were higher in all inoculated microcosms with invertebrates than in inoculated microcosms without invertebrates (Fig. 1A–C). However, the magnitude of enhancement depended on the invertebrate species. *Chironomus riparius*, *L. variegatus*, and *Tubifex* spp. increased LOI (Fig. 1A) and DOC (Fig. 1B) by 60 to 400% and increased bacterial activity by 30 to 600% relative to inoculated controls without invertebrates. *Asellus aquaticus* and *G. pulex* processed, on average, 60% more detritus than the other invertebrate species (Fig. 1A, B) and sustained 50 to 500% higher bacterial activity than the other invertebrate species (Fig. 1C).

RISA banding patterns were variable, and the bands derived from inocula were overlaid by bands derived from invertebrate-associated bacteria. That is, bands appeared in microcosms with invertebrates that were not visible in microcosms containing only inocula. Approximately 50 to 60% of the bacterial community in microcosms with *Tubifex* spp., *L. variegatus*, and *C. riparius* and 30 to 35% of the bacterial community in microcosms with *A. aquaticus* and *G. pulex* originated from the invertebrates. In the cluster analysis (Fig. 2), bacterial communities in sterile microcosms with invertebrates were not necessarily separated from bacterial communities in inoculated microcosms with the same invertebrate species (ANOSIM,  $p > 0.4$ ; Fig. 2). Bacterial commu-

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Bars with the same letters are not significantly different (1-way analysis of variance, Tukey's Honestly Significant Difference post hoc test,  $n = 7$ ,  $p < 0.05$ ).

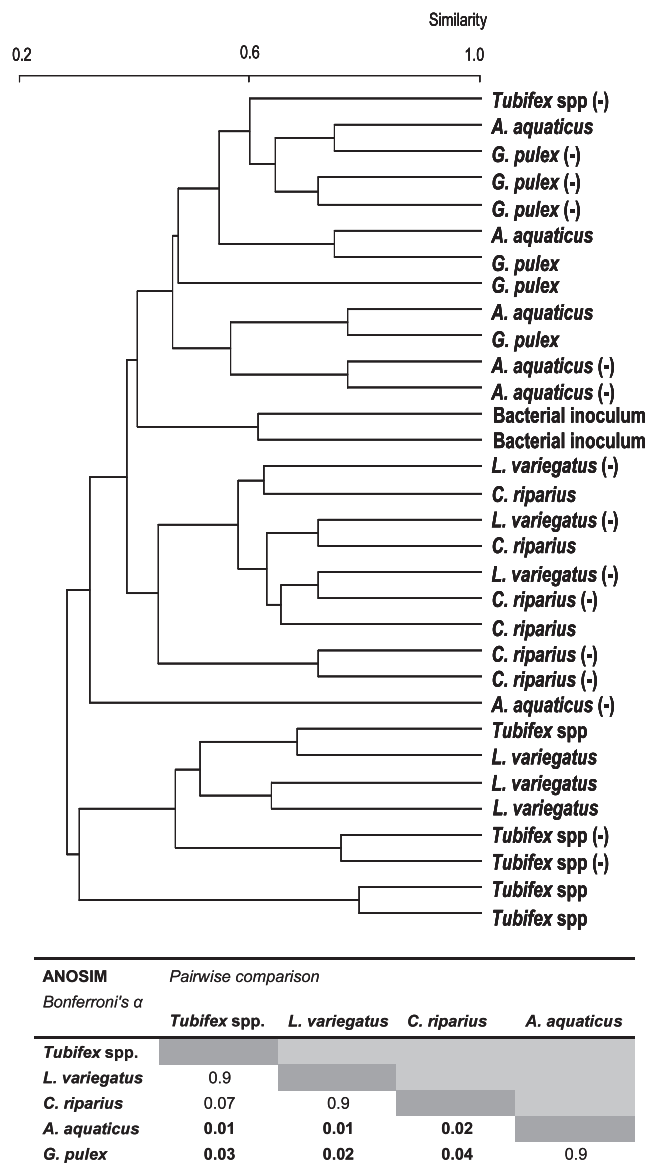


FIG. 2. Jaccard-based dendrogram representing level of similarity between bacterial community structure in inoculated microcosms with *Asellus aquaticus*, *Gammarus pulex*, *Chironomus riparius*, *Tubifex* spp., or *Lumbriculus variegatus* ( $n = 3$ /invertebrate treatment), sterilized microcosms with different invertebrate species (-), and inoculated microcosms without invertebrates (Bacterial Inoculum). The results of a Jaccard-based analysis of similarity (ANOSIM) and pairwise comparisons with Bonferroni-correction are shown below the dendrogram. Bold indicates statistically significant differences ( $p < 0.05$ ) between the microbial communities in pairs of invertebrate treatments.

nities in microcosms with *G. pulex* and *A. aquaticus* clustered together, but differed (ANOSIM,  $p < 0.05$ ) from bacterial communities in microcosms with *Tubifex* spp., *L. variegatus*, and *C. riparius*.

Development of sediment Eh profiles over time differed between inoculated microcosms with and without invertebrates (see Fig. 3 for 1 representative replicate of each treatment). Replicates were very similar within all invertebrate treatments. Averaged Eh at each depth differed among invertebrate treatments (ANCOVA,  $F = 60.05$ ,  $p < 0.05$ ). Eh profiles differed between microcosms with sediment-dwelling biodiffusors (*A. aquaticus* and *G. pulex*) and microcosms with burrowing invertebrates (*Tubifex* spp., *L. variegatus* and *C. riparius*) (Tukey HSD test; Fig. 4). Eh was higher at the sediment-water interface in microcosms with *G. pulex* and *A. aquaticus* than in microcosms with *Tubifex* spp., *L. variegatus*, and *C. riparius* (Fig. 4). *Chironomus riparius* initially moved near the electrode, but then constructed burrows away from the electrode, which subsequently resulted in stratification of the sediment Eh near the electrode (Fig. 3). Eh in subsurface layers of the sediment increased in microcosms with *Tubifex* spp. and *L. variegatus* (Fig. 3). However, overall Eh was lower in microcosms with burrowing invertebrates than in microcosms with sediment-dwelling *G. pulex* and *A. aquaticus* and in inoculated microcosms without invertebrates (Fig. 4).

### Discussion

Invertebrates strongly contributed to bacterial activity and detritus processing and overruled effects of the starting bacterial inocula. Enhancement of bacterial activity and detritus processing by invertebrates has been observed in a number of studies (van de Bund et al. 1994, Wieltchnig et al. 2008, Hunting and van der Geest 2011). Detritivorous invertebrates incorporate large amounts of bacterial biomass, but this loss is often (over-) compensated by the stimulatory effects of nutrient excretion, partial degradation of organic matter, and irrigation in the presence of invertebrates (Traunspurger et al. 1997, Meysman et al. 2006). Similar mechanisms probably were responsible for the stimulatory effects observed in our study.

The magnitude of bacterial activity and detritus processing depended on invertebrate bioturbation/feeding trait, and the presence of sediment-dwelling biodiffusors resulted in higher bacterial activity and detritus processing than did the presence of burrowing organisms. This result differs from the outcomes of other studies (Mermillod-Blondin and Rosenberg 2006, Meysman et al. 2006) in which burrowing organisms were identified as the main bioturbators affecting organic matter processing in sediments. This discrepancy may be a consequence of differences in invertebrate densities among studies. Other investi-

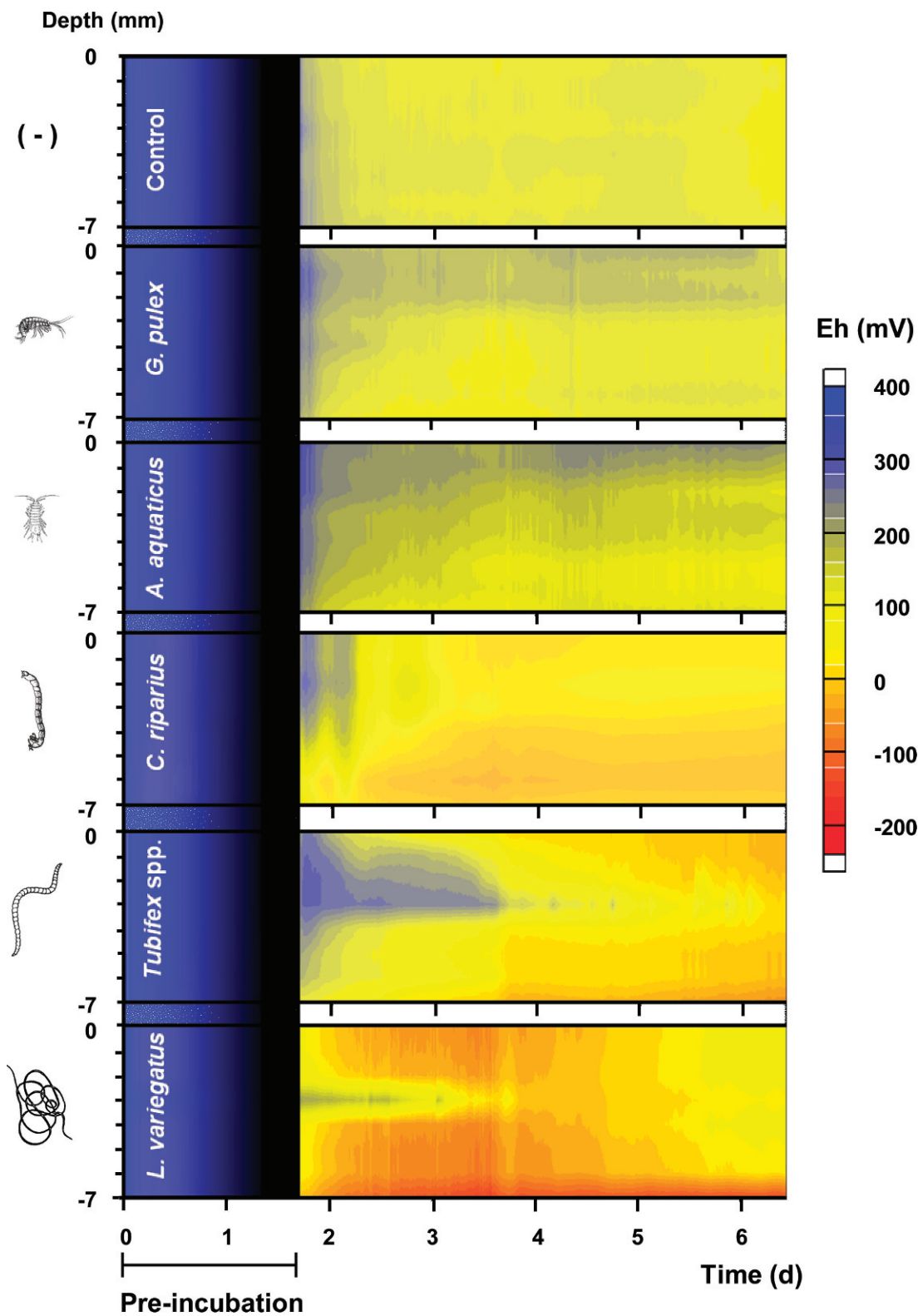


FIG. 3. Redox potential (Eh) profiles in depth (7 mm) and time of incubation in microcosms with *Gammarus pulex*, *Asellus aquaticus*, *Chironomus riparius*, *Tubifex* spp., or *Lumbriculus variegatus* compared to in inoculated microcosms without invertebrates (-). The contour plots are representative of 3 replicates for each treatment.



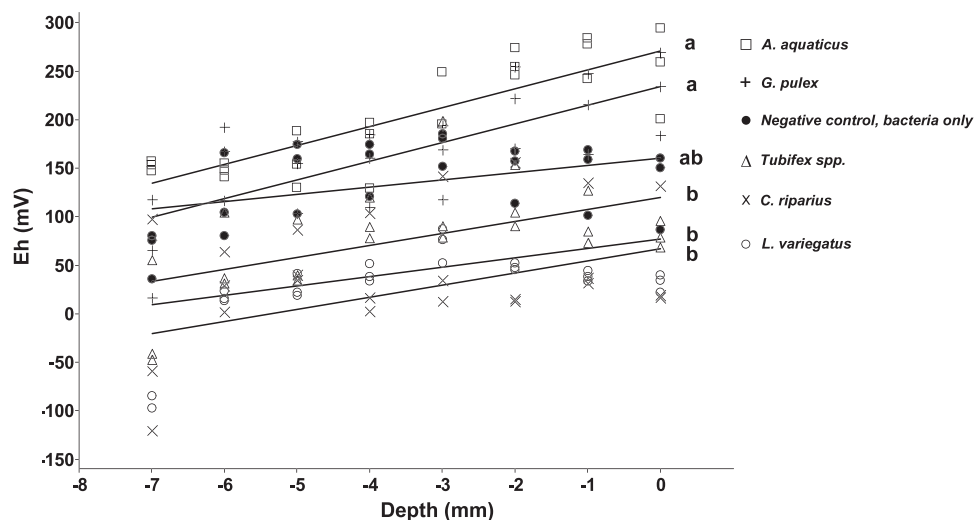


FIG. 4. Mean ( $n = 3$ ) redox potential (Eh) at each depth in microcosms with *Asellus aquaticus*, *Gammarus pulex*, *Chironomus riparius*, *Tubifex* spp., or *Lumbriculus variegatus* compared to in inoculated microcosms without invertebrates (control). Depth dependence was defined by the slopes and did not differ significantly among treatments (general linear model–analysis of covariance (GLM–ANCOVA,  $p = 0.14$ ). Therefore, we excluded Eh  $\times$  depth interactions and detected invertebrate treatment effects with a Tukey's Honestly Significant Difference post hoc test ( $p < 0.05$ ). Lines with the same letter are not significantly different.

gators used natural densities of invertebrates. Oligochaetes and *C. riparius* often are far more abundant (100:1) than *A. aquaticus* and *G. pulex* in natural systems, resulting in an  $\sim 25\times$  difference in relative abundances of burrowing and sediment-dwelling invertebrates between our model system and those in other studies. Thus, density appears to influence invertebrate effects on sediment processes. This result highlights the need for standardization by invertebrate mass or biovolume (sensu Michaud et al. 2005) when characterizing invertebrate communities. In addition, we focused on the top layer (25 mm) of the sediment. The burrowing organisms used in our study often act in deeper layers of natural sediments, and these effects were not captured in our microcosms. Thus, the relative contribution of burrowing organisms to decomposition may have been underestimated in our microcosms.

Bacterial community structure was strongly affected by invertebrate bioturbation/feeding traits, i.e., bacterial communities differed between microcosms with sediment-dwelling invertebrates and those with burrowing species. Part of the bacterial community (up to 50%) originated from the invertebrates, a result suggesting that bacteria introduced by invertebrates strongly affected bacterial community structure and functioning. In addition, similarity in microbial community structure mirrored similarity in sediment Eh conditions. Eh measurements reflect an ensemble of system-specific redox reactions, so how Eh values

obtained in our study are related to conditions in natural sediments is not known. Nonetheless, our results suggest that invertebrate bioturbation/feeding activities shape redox conditions and microbial community structure.

Only a few investigators have addressed the effect of habitat heterogeneity and the presence of macroinvertebrates on microbial diversity in sediments. Burrow walls might harbor unique microbial consortia because physicochemical conditions in burrows are generally more stable than those at the frequently disturbed sediment surface (Kristensen and Kostka 2005). Papaspyrou et al. (2005, 2006) reported results that support this hypothesis, but evidence is increasing that similarity in bacterial community structure and metabolic activity coincide with similarity in geochemical conditions, in particular redox conditions (Bertics and Ziebis 2009, Hunting and van der Geest 2011), as observed in our study.

Our results suggest tight coupling between invertebrate bioturbation/feeding activities and redox conditions, microbial communities, and detritus processing. Hence, our findings support the proposal that aquatic invertebrate functional traits are linked to ecosystem processes (Hillebrand and Matthiessen 2009, Nogaro et al. 2009). A key issue in trait-based approaches to research on biodiversity–ecosystem function is identifying and quantifying those functional traits relevant to ecosystem properties (Naeem and Bunker 2009). We were able to use Eh profiles to

provide quantitative signatures that demonstrated the effect of invertebrate activity on biogeochemical processes and detritus processing. We showed that invertebrates with different functional traits had very different effects on spatial and temporal properties of sediment Eh. More important, invertebrates with similar bioturbation/feeding traits produced comparable Eh profiles in space and over time. Eh profiles produced by sediment dwellers (*A. aquaticus* and *G. pulex* [biodiffusers]) and burrowing organisms (*Tubifex* spp., *L. variegatus* [upward conveyors] and *C. riparius* [gallery diffusers]) differed strongly. Moreover, Eh profiles produced by upward conveyors and gallery diffusers also differed. Therefore, we propose using Eh profiles as readily obtained footprints of invertebrate bioturbation/feeding activities.

How ecosystem functional responses observed in our model system might change in multispecies invertebrate assemblages or under natural conditions is not clear. However, the differing effects of invertebrates with different bioturbation/feeding activities on bacterial community structure and detritus processing suggest that these traits could be used to study effects of faunal diversity on ecosystem processes in fresh water, as well as in marine (Covich et al. 2004, Solan et al. 2004, Birchenough et al. 2012) and grassland ecosystems (Klump and Soussana 2009, Mulder and Elser 2009). We showed that aquatic invertebrate bioturbation/feeding traits mediate detritus processing, redox geochemical characteristics of the sediment, and microbial community structure. Our results support the trait-based framework linking bioturbation to ecosystem properties and processes in a wide range of habitats (Mermillod-Blondin 2011).

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