Shedding light on detritus: Interactions between invertebrates, bacteria and substrates in benthic habitats

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Shedding Light on Detritus

Interactions between Invertebrates, Bacteria and Substrates in Benthic Habitats

Ellard R. Hunting

PhD Thesis, University of Amsterdam, 2013

Invitation

To the public defense and reception on Wednesday September 25th, 12:00h, in the Agnietenkapel, Oudezijds Voorburgwal 231, Amsterdam

Paranimphs:

Coen Wagner
Louwrens Wendelaar
Bonga

Ellard R. Hunting
Shedding Light on Detritus:

*Interactions between Invertebrates, Bacteria and Substrates in Benthic Habitats*

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The research presented in this thesis results from a collaborative effort of the National Institute for Public Health and the Environment (RIVM) and the University of Amsterdam on “Interacting consortia of detritus feeding invertebrates and micro-organisms: towards a linkage of species composition and functional parameters for aquatic communities”, and was financed by the National Institute for Public Health and the Environment (RIVM).
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I began to read, and promptly I wondered whether I was reading written lines, or seeing visions

V. Nabokov, Despair
Chapter 1

General introduction
The processing of dead organic matter, also known as detritus, is a central ecosystem process in aquatic habitats driven by detritus feeding organisms that are mostly located at the bottom of water bodies where dead organic matter (OM) accumulates (Webster and Benfield 1986, Graça 2001). These benthic communities are composed of invertebrates, fungi and bacteria, living in, on, or near submerged substrates, and these organisms interact with each other and their substrate in a number of ways. Firstly, facilitative interactions occur among organisms. Although invertebrates are known to consume large amounts of bacterial biomass (Hall and Meyer, 1998), bacteria can also benefit from the presence of invertebrates. For instance, invertebrate litter consumption facilitates the penetration of bacteria into otherwise inaccessible leaf tissue (e.g. De Boer et al., 2005), and sediment reworking by invertebrates can alter sediments by modifying texture, distributing solid particles, and introducing O\textsubscript{2} into otherwise anoxic zones (Meysman et al., 2006; Covich et al. 2004, Navel et al. 2010). Invertebrates, on the other hand, can also benefit from the presence of microorganisms. Microbial conditioning of leaf litter increases palatability (Graça, 2001; Canhoto and Graça, 2008), and the production of complementary enzymes or useful carbon products also contributes to a wider use of resources (e.g. Osono, 2007), and detritivorous invertebrates particularly can benefit from this increased diversity in resources (e.g. Lavelle et al., 1998; Hall and Meyer, 1998).

Another important attribute of detritus is that it can vary widely in chemical composition, even within a given plant species (Cornwell et al., 2008; Lecerf and Chauvet, 2008). It is well known that some types of organic matter can easily be utilized (labile carbon), whereas others are nutrient-poor or contain high concentrations of aromatic compounds that are resistant to degradation (recalcitrant carbon) or detrimental to microbial and invertebrate consumers (e.g. Gessner and Chauvet, 2002; Hladyz et al., 2009). Changes in the chemical composition and diversity of OM can therefore entail changes in OM processing by both microorganisms and invertebrates.

Differences in benthic biodiversity and composition of organic matter can thus become visible in the OM processing, and understanding the significance of biodiversity, as well as the mechanistic basis behind effects of biodiversity and OM composition, is essential to assess the consequences of biodiversity change for organic matter processing (Gessner et al., 2010). Biodiversity has long been assessed by characterizing or manipulating species composition, and a number of studies demonstrated that higher levels of biodiversity support higher OM processing rates (Johnsson and Malmqvist 2000; Bell et al., 2005). However, increasing evidence suggest that a functional characterization of communities, i.e. characterizing and sorting species according to the traits
that likely affect ecosystem processes (e.g. body size, feeding strategy, locomotion), is likely more valuable in explaining links between biodiversity and OM processing as species with similar functions will unlikely have complementary effects on organic matter processing (e.g. Reiss et al., 2009).

Although it is likely that links between benthic biodiversity and OM processing are driven by similar mechanisms across different ecosystem types (forest floors, stream beds, coral reefs) (Gessner et al., 2010), it remains a challenge to identify general drivers of decomposition in distinct benthic detrital food webs.

**Soft sediments and hard substrates**

Two distinct types of substrates, hard substrates and soft sediments, characterize the benthic environment, and these substrates differ in a number of ways. Firstly, they are typically recognized to support different groups of organisms. Shredders, collectors and deposit feeders (e.g. crustaceans, dipterans and oligochaetes), that actively seek and feed on particulate organic matter, abound in soft sediments, while sessile filter feeders (e.g. sponges and bivalves) that feed on particulate and dissolved organic matter dominate hard substrates. Secondly, while mobile invertebrates in soft bottom sediments can move towards more favorable conditions, sessile invertebrates attached to hard substrates are not able to respond to changes in environmental variables, and hence it is likely that abiotic variables more strongly affect hard substrate invertebrate communities as compared to soft bottom sediments, pointing to a potential difference in magnitude of the interactions between the abiotic and biotic components of these distinct substrates. Studying simultaneously a soft bottom sediment system and a hard substrate system would thus provide the unique opportunity to study distinct underexposed aspects of the interaction between the (functional) composition of invertebrate and bacterial communities, organic matter processing and abiotic variables in benthic systems. This thesis therefore focused on the interactions between bacteria, invertebrates and their substrates in two distinct ecosystems: soft bottom sediments and hard bottom substrates, in which gaps in our current understanding of the interactions between abiotota, biota and inherent OM processing are identified for both substrates.

*Soft bottom sediments*

Soft bottom sediments harbor a diverse invertebrate community that is composed of species that are widely distributed. Because up to 50% of the particulate organic matter (POM) produced in aquatic ecosystems
becomes trapped in subsurface sediments (Herbst, 1980; Metzler and Smock, 1990), the effect of bioturbation on organic matter processing gained increased interest (Mermillod-Blondin and Rosenberg, 2006; Nogaro et al., 2009). Macro-invertebrates alter sediments by modifying texture, distributing solid particles, and introducing O$_2$ into otherwise anoxic zones (Meysman et al., 2006; Covich et al. 2004, Navel et al. 2010). Hence, biological and geochemical components of subsurface sediments might be coupled, suggesting that differences in invertebrate community composition can become visible in ecosystem processes, although this suggestion requires experimental validation.

Species-specific sensitivities to abiotic constraints such as temperature, solar radiation, pH, salinity limit the occurrence of organisms to certain environments (e.g. Bervoets et al., 1996). Likewise, anthropogenic disturbances like eutrophication and environmental pollution may also alter and reduce the diversity of microorganisms and detritivorous invertebrates (e.g. Kiffney et al., 1994; Loayza-Muro et al., 2010; Santos et al. 2012). Moreover, at sub-lethal levels, both natural and human induced abiotic pressure can alter the locomotion and behavior of aquatic invertebrates (Van der Geest et al., 1999; Maltby et al., 2002; Brooks et al., 2009; Bundschuh et al., 2012). It is likely that links between invertebrate bioturbation/feeding behavior and ecosystem functioning are affected by environmental stressors. However, stress responses are traditionally studied on the species level, while perturbations of functionality (e.g. animal behavior) can cascade toward ecosystem processes and therefore ecologically much more relevant, but virtually unknown. Likewise, solar radiation is an important abiotic variable that specifically may affect bacterial communities (e.g. Baldy et al., 2002; Piccini et al., 2009; Zepp et al., 2011). Solar radiation, and especially UV radiation (280-320 nm) is known to have detrimental effects on DNA (e.g. Santos et al., 2012a), or change the chemical composition and palatability of organic compounds by photodegradation (e.g. Engelhaupt et al., 2002; Sulzberger and Durisch-Kaiser, 2009). Such changes in the chemical composition of OM may subsequently cascade toward shifts in bacterial community composition due to the interplay between bacterial resource niches (i.e. the type of substrates that are utilized) and available resources (e.g. Salles et al., 2009). However, the effects of solar radiation on bacterial communities residing in sediments remain completely unexplored.

The significance of invertebrate species composition for detritus processing is a matter of intense debate. However, widely different measures are used to identify detritus processing, suggesting a scientific dilemma. Currently, detritus processing is typically evaluated with a number of functional parameters. These include bacterial activity (Mermillod-Blondin and Rosenberg, 2006; Nogaro et al., 2009) and
(functional) diversity (Bertics and Ziebs, 2009; Salles et al., 2009; Gravel et al., 2011), the geochemical characteristics of the sediment (Mermillod-Blondin et al., 2002; Solan et al., 2004; Meysman, 2006; Birchenough et al., 2012) and measures of cellulose decomposition (Boulton and Quinn, 2000; Tiegs et al., 2008; Young et al., 2008; Imberger et al., 2010). However, these functional parameters are rarely studied simultaneously, and therefore the relative importance, reliability and cohesion remain uncertain. Experiments are thus required that test the predictive potential of invertebrate functional metrics in relation to OM processing.

**Hard substrates: Mangrove roots**

A great diversity of hard substrates can be found in continental waters and seas. Bedrock and coarse mineral debris (gravel and boulders) offer a habitat for detritus feeders, but organic substrates such as wood debris in rivers and tree roots in riparian and coastal habitats are important stimulants for detritus processing, as detritus accumulation around extensive root systems may also produce organic substances in situ that can directly affect the associated fauna.

For the present study roots of the mangrove *Rhizophora mangle* were selected. Mangroves form the dominant vegetation in tidal, saline wetlands along (sub-)tropical coasts (Chapman, 1976; Tomlinson, 1986). Submerged portions of mangrove aerial roots are dominated by sponges (Sutherland, 1980; Ellison and Farnsworth, 1992). Sponges are efficient filter feeders and mangrove associated sponges primarily feed on mangrove-derived particulate matter and dissolved organic matter (Granek et al., 2009).

Mangrove-derived OM, originating from decaying leaves and leachates from mangrove roots, is composed mainly of tannins and polyphenolic compounds (Maie and Jaffe, 2006), in which concentrations of tannins and polyphenols may vary depending on tissue, growth stage, and environmental conditions (Northup et al., 1998; Lin et al., 2006). Tannins are a group of secondary metabolites that is known for their anti-microbial and anti-herbivore activity (Cameron and LaPoint, 1978; Alongi, 1987; Scalbert, 1991; Arnold and Targett, 2002, Erickson et al., 2004), and it may adversely affect associated macrofaunal abundance (Lee, 1999). Since mangrove sponges mainly feed on mangrove-derived organic matter (Ellison and Farnsworth, 1996; Granek et al., 2009), it is possible that mangrove-derived DOM influence sponge physiology or larval settlement, and subsequently alters species distributions.

The composition of mangrove-associated sponges is relatively species poor, heterogeneous, and very distinct from sponge communities living on connected, nearby reefs (e.g. Van Soest, 1978, 1980, 1984; Wulff, 2004). The mechanisms that underlie this distinction remain uncertain to date.
(for review see: Wulff, 2012). Transplantation of typical reef species to mangrove roots results in the rapid deterioration of the transplanted sponges (Farnsworth and Ellison, 1996; Wulff, 2004; Pawlik et al., 2007). It has therefore been argued that abiotic factors (such as salinity; wave exposure; particle suspension) are of prime importance for sponge survival and perseverance in mangroves ecosystems, but it remains uncertain which abiotic factor is the key controlling variable (Pawlik et al., 2007; Wulff, 2012).

Sponges form close associations with symbiotic microorganisms. Various molecular studies have demonstrated that sponges host a diverse, and largely host specific symbiotic community (e.g. Taylor et al., 2007, and references therein), although the ecological and evolutionary nature of these communities remains uncertain to date (Thacker and Freeman, 2012). Evidence is now accumulating that sponge-hosts obtain carbon and other nutrients from their microbial associates (De Goeij et al. 2008a,b; Freeman and Thacker, 2011; Ribes et al. 2012), although the identity of key compounds and bacterial metabolic pathways remain completely unresolved (Thacker and Freeman, 2012). Increasing evidence also suggests that only a limited number of bacterial and fungal species are able to degrade complex polyphenols and tannins (Bhat et al., 1998, and references therein). It is thus possible that an interaction between recalcitrant compounds derived from mangroves and the presence of bacterial symbionts capable of degrading mangrove-derived DOM plays a pivotal role in the observed differences in species composition between mangrove and reef sponge communities. Responses of sponge-bacterial consortia to the mangrove root substrate and mangrove-derived (D)OM thus requires further evaluation.

**Aim and objectives**

Studying simultaneously a soft bottom sediment system and a hard substrate system provides the unique opportunity to study different underexposed aspects of the interaction between the (functional) composition of invertebrate and bacterial communities, and organic matter processing. Would it be possible to identify general drivers of detritus processing among distinct benthic ecosystems? The aim of this thesis was therefore to unravel interactions between the (functional) composition of invertebrate and bacterial communities, organic matter processing and abiotic variables in two contrasting benthic detrital food webs: one on soft bottom sediments and one on solid substrate ecosystems. To this purpose, the following objectives have been set:

- To evaluate the impact of OM composition on invertebrate-substrate interactions and organic matter processing
To assess the impact of abiotic stressors on invertebrate-substrate interactions and organic matter processing
To quantify the effect of functional diversity of bacteria and invertebrates on organic matter processing

Thesis outline

To meet the objectives of the present study, two series of experiments were performed on organic matter processing in two contrasting benthic habitats.

**Part 1 – Invertebrate-substrate interactions in soft bottom sediments**

The first set of experiments focused on invertebrate-substrate interactions in soft bottom sediments. Evaluating the importance of invertebrate functional diversity, especially their bioturbation behavior, for bacterial communities and detritus processing, requires manipulation of the invertebrate community, and therefore these experiments were performed in laboratory microcosms and outdoor mesocosms.

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. Chapter 2 therefore evaluated whether bioturbation/feeding strategies of aquatic invertebrate species differentially affect detritus processing and benthic microbial community structure and tested the utility of redox potential (Eh) profiles as biogeochemical signatures of the types of bioturbator species in laboratory microcosms.

Since chemical stressors may decouple links between biodiversity and ecosystem processes, Chapter 3 evaluated how toxicants affect the functional links between invertebrate bioturbation and ecosystem functioning. To this purpose, the effects of the model toxicant copper on two functionally distinct macrofauna species (*Asellus aquaticus* and *Tubifex* spp.), detritus processing and microbial activity and metabolic diversity were determined in 5–day microcosm experiments. The effect of altered locomotion and activity and reduced bioturbation were assessed with spatio-temporal redox (Eh) profiles in the upper sediment layer.

Those who study biodiversity effects on OM processing would benefit from standardized ways of measuring detritus processing. One standardized proxy with a chemical composition that can be easily adjusted for experimental purposes is required. Therefore, in Chapter 4 the performance of a novel decomposition and consumption tablet (DECOTAB) consisting of cellulose powder embedded in an agar matrix to evaluate microbial decomposition and invertebrate feeding was tested.
This chapter describes the preparation of the newly developed DECOTABs and evaluates some potential applications in laboratory microcosms and outdoor mesocosms.

Potential effects of solar radiation on bacterial communities residing in sediments remain completely unexplored. Chapter 5 investigated the effect of solar radiation on the functional composition of bacterial communities in shallow aquatic sediments, and compared the effect of light and dark incubation on bacterial metabolic diversity in sediment microcosms containing two different substrates: recalcitrant peat and palatably fresh plant biomass.

Several parameters are at hand to quantify the decomposition process, but these are seldomly studied in coherence and mainly rely on laboratory experiments. Chapter 6 tested therefore whether several functional parameters measured in multispecies invertebrate assemblages in outdoor mesocosms could be predicted from their responses to single invertebrate species experiments under laboratory conditions. To this purpose, bacterial functional diversity and activity, sediment redox potential and DECOTAB mass loss were measured in laboratory microcosms and outdoor mesocosms in the presence of single invertebrate species and manipulated multi-species assemblages.

**Part 2 – Sponge-environment interactions in mangrove stands**

The second set of experiments focused on the role of root substrate as driver of sponge community composition in mangrove ecosystems. Experiments were performed in a tropical mangrove ecosystem and an adjacent reef.

Chapter 7 aimed to quantify the diversity of mangrove associated sponges in the inner bays of Curaçao and Aruba, and explored correlations between a set of physico-chemical variables and sponge distributions. Tannin concentrations of selected mangrove roots were compared to sponge cover and tested as a possible driver of local heterogeneity. A positive relationship between sponge coverage and tannin concentrations in roots was observed, but the reason for this observation remained uncertain. The objective of Chapter 8 was therefore to evaluate whether tannins play a role in sponge recruitment and whether mangrove roots enhance production of tannins and total phenolics in response to sponge colonization. These aspects were addressed by performing in situ recruitment and translocation experiments.

Chapter 9 tested the hypothesis that tannin-degrading microorganisms within the endobiontic community of mangrove sponges may be partly responsible for the structural differences in reef and mangrove sponge communities. To test this assumption, the presence of tannin-degrading organisms in a random set of species collected from mangrove roots and a
nearby reef by assaying tannase activity and evaluated whether endobionts were able to grow on artificial substrate containing mangrove root extracts was explored qualitatively.

To discriminate the role of habitat, substrate and symbiotic bacteria in driving sponge distributions, Chapter 10 monitored survival and condition of typical mangrove and reef sponge species after in situ reciprocal transplantation to DOM-releasing mangrove roots and DOM-free surrogate roots (PVC tubes) in both mangrove and reef environments. Next, the structure and stability of the symbiotic bacterial community in the sponge host before and after transplantation were determined. Finally, the carbon utilization patterns of the symbiotic bacterial communities of the individual sponge species were analyzed.

Chapter 11 tested whether mangrove-DOM leachates from roots are responsible for the observed deterioration of reef species transplanted to mangrove roots. To this end, a typical reef species and a typical mangrove species were transplanted to mimicry substrates containing mangrove root extract and to control substrates without extract. Mangrove DOM was also injected directly into tissues of both sponge species.

Finally, Chapter 12 evaluated the interaction between biotic and abiotic components of two contrasting benthic detrital food webs and attempted to identify general drivers of OM processing in benthic detrital food webs.
PART I

Invertebrate-substrate interactions in soft bottom sediments
Chapter 2

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

Published as:
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, which is 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\textsubscript{2} 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 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 may be categorized by the way they affect the physicochemical and biogeochemical properties of the sediment (François et al. 1997, Gerino 2003, Nogaro et al. 2009). Invertebrates may act as: 1) biodiffusors 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, Bircheneough et al. 2012). Spatial and temporal redox potential 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). DWS contains 200 mg CaCl₂·2H₂O, 180 mg MgSO₄·7H₂O, 100 mg NaHCO₃, and 20 mg KHCO₃/L demineralized H₂O (pH 8.1, hardness 210 mg/L CaCO₃, 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 brasilense, Bacillus subtilis, Paenibacillus polymyxa, Pseudomonas putida, Sphingomonas paucimobilis, Micrococcus luteus, Streptomyces antibiotica, Pseudomonas stutzeri, Flavobacterium sp., Aeromonas salmonida, Paracoccus pantotrophus*, and *Aminobacter aminovarans* (obtained from the Fungal Biodiversity Centre, CBS-KNAW, Utrecht, The Netherlands), all grown in brain–heart broth (Merck, Darmstadt, Germany) and peptonised milk nutrient (Sigma-Aldrich, St. Louis, United States) (ratio 100:15). We standardized bacterial biomass for each strain by dilution to obtain an optical density at 600 nm (OD600) 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 Nogara et al. (2009). The isopod *Asellus aquaticus* and the amphipod *Gammarus pulex* are omnivorous sediment dwellers that act as biodiffusors, i.e., grazing the upper layer of detritus and biofilms on sediment particles. Larvae of the nonbiting 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 ± SD, 0.35 ± 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 redox potential.

**Organic matter processing**

We characterized detritus processing as sediment mass loss on ignition (LOI) and increase in DOM 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 DOM 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, United States) 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 μL 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°C until analysis. We extracted bacterial DNA from 2 g of sediment using PowerSoil extraction kits (Mo-Bio, Carlsbad, United States). 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.
Fig. 1. Mean (±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 Asellus aquaticus, Gammarus pulex, Chironomus riparius, Tubifex spp., and Lumbriculus variegatus treatments in microcosms with bacterial inocula, without bacterial inocula, and in controls (inoculated microcosms without invertebrates). 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).
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, 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 the mean Eh in time at each depth, and depth was the covariate. In the ANCOVA procedure, 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 DOM (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 communities 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*. 

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.
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 redox potential 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, Wieltschnig et al. 2008, Hunting and van der Geest 2011). Detritivorous invertebrates incorporate large amounts of bacterial biomass, but this loss is often (over-) compensated for 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 et al. 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 investigators 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 ~25× difference in relative abundances of burrowing and sediment-dwelling invertebrates.
Fig. 3. Redox potential (Eh) profiles in depth (7 mm) and time of incubation in microcosms with Asellus aquaticus, Gammarus pulex, 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.
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

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 × 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.
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 [biodiffusors]) and burrowing organisms (Tubifex spp., L. variegatus [upward conveyors] and C. riparius [gallery diffusors]) 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 (Solan et al. 2004, Covich 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).
Chapter 3

Effects of copper on invertebrate-substrate interactions
**Abstract:** Toxic pressure may alter bioturbation activities of aquatic invertebrates, thereby potentially affecting the links between invertebrate community structure and ecosystem functioning. This study therefore aimed to evaluate how toxicants affect invertebrate bioturbation and decomposition. To this purpose, the effects of copper on functionally distinct macrofaunal species (*Asellus aquaticus* and *Tubifex* spp.), decomposition (DOC) and microbial activity (AMR) and metabolic diversity (CMD) were determined in 5–day microcosm experiments. Invertebrate bioturbation was assessed with redox potential (Eh) profiles within the sediment. Concentration-response curves of the functional parameters DOC, AMR and CMD in the presence of *Tubifex* spp. were similar to the concentration-response curve of *Tubifex* spp. survival and resulted in comparable EC_{50} values. In contrast, the EC_{50} values of the functional parameters DOC, AMR and CMD in the presence of *A. aquaticus* are significantly lower than the EC_{50} of *A. aquaticus* survival. Sediment redox potential profiles showed that this was caused by a reduced interaction between *A. aquaticus* and the sediment at sub-lethal copper concentrations. This points to a decoupling of invertebrate community structure and ecosystem functioning under stress, suggesting that functional parameters (e.g. decomposition), compared to structural parameters, may serve as more sensitive and reliable parameters for assessing ecological water quality and ecosystem functioning.

**Keywords:** Copper, Bioturbation, Decomposition, Ecosystem Functioning, Sediment, Invertebrates.
Decomposition of detritus is a vital ecosystem process driven by both microorganisms and invertebrate detritivores (Webster and Benfield 1986, Graça 2001, Gessner et al. 2010, Tank et al. 2010). Ecosystems are, however, under continuous toxic pressure, directly affecting decomposer organisms and therewith indirectly the ecosystem process they provide. However, to which extent toxic pressure on decomposing organisms cascades toward disordering ecosystem processes remains largely unknown.

Since a large part (>50%) of the detritus becomes trapped in subsurface sediments (Herbst 1980, Metzler and Smock 1990), the effect of bioturbation by invertebrates on organic matter processing gained increased interest (Mermillod-Blondin and Rosenberg 2006, Nogaro et al. 2009). It has indeed been demonstrated that detritus processing is largely influenced by invertebrate consumption and bioturbation activities that promote microbial decomposition by altering sediment texture, distributing solid particles, and introducing $O_2$ into otherwise anoxic zones (Meysman et al. 2006, Navel et al. 2010, Hunting and Van der Geest, 2011). Consequently, decomposition is directly related to the functional composition of invertebrate communities (François et al. 1997, Gérino et al. 2003, Mermillod-Blondin 2011, Hunting et al., 2012). However, toxic pressure may alter the locomotion and behavior of aquatic invertebrates (e.g. Van der Geest et al., 1999; Maltby et al., 2002; Brooks et al., 2011), potentially affecting these links between invertebrate community structure and ecosystem functioning. Therefore, the aim of the present study was to evaluate how toxicants affect the functional links between invertebrate bioturbation and ecosystem functioning. To this purpose, the effects of the model toxicant copper on functionally distinct macrofauna species (*Asellus aquaticus* and *Tubifex* spp.), detritus processing and microbial activity and metabolic diversity were determined in 5–day microcosm experiments. Altered locomotion and activity and reduced bioturbation were assessed with spatio-temporal redox (Eh) profiles within the upper sediment layer (Hunting et al., 2012).

**Methods**

**Test organisms and test compound**

Two aquatic invertebrate species were chosen to represent distinct types of sediment reworking. The oligochaete *Tubifex* spp. is an upward conveyor, i.e., deposit feeders that create burrowing networks in the sediment and defecate on the sediment surface. The isopod *Asellus aquaticus* is an omnivorous sediment dweller that acts as biodiffusor, i.e., grazing the upper layer of detritus and biofilms on sediment particles. *Tubifex* spp. was obtained from a laboratory culture. *Asellus aquaticus* was
collected from nearby pristine ponds. Copper, an essential metal and common pollutant, was used as a model chemical stressor.

**Microcosm preparation and sediment spiking**

We constructed microcosms from 50-mL glass vials (25-mm diameter) filled with fine-grained, ignited quartz sand as mineral substrate (17 g/vial, grain size: 0.1–0.5 mm, sediment depth: 18 mm). The sediment was spiked with the following nominal copper concentrations (CuCl2.2H2O, copper standard, Fluka): 0 (control), 10, 20, 50, 100, 200, and 500 mg/kg dry weight. Appropriate amounts of copper stock solution were added to 420 g wet sediment in 1-L glass bottles. Treatments that required less or no metal stock solution were supplemented with deionized water, so equal volumes were added to all treatments. Freeze-dried, ground, and sieved stinging nettle (*Urtica dioica* L., ≤500 mm particle size, 365 mg) was added as detritus. The bottle was placed for 24 h on a roller bank (20 rpm) in order to homogenize the food-metal-sediment mixture, after which it was divided over the replicate (n=7 per treatment) microcosms (17 g/microcosm). Microcosms were carefully topped up with 35 mL of Dutch Standard Water (deionized water with 200 mg/L CaCl$_2$*2H$_2$O, 180 mg/L MgSO$_4$*H$_2$O, 100 mg/L NaHCO$_3$, and 20 mg/L KHCO$_3$; hardness is 210 mg as CaCO$_3$/L and pH 8.2). After settling of the sediment, microcosms were gently aerated and conditioned for one week, allowing copper to equilibrate with the sediment and a stable sediment layer to be formed.

**Toxicity test**

To evaluate the influence of copper on invertebrate survival, a five-day toxicity experiment was performed. Microcosms were kept at 20 ± 1 °C and were constantly aerated. The experiment consisted of three invertebrate treatments per copper range: 1) a control treatment without invertebrates; 2) microcosms with 2 specimens of *A. aquaticus* and 3) microcosms with 8 specimens of *Tubifex* spp., thereby containing equal invertebrate dry mass for the invertebrate treatments (Hunting et al., 2012). Each invertebrate treatment – copper concentration combination was replicated seven times. The experiments were initiated by introducing the invertebrates to the microcosms. At the end of the experiment (day 5), the sediment was sieved through a 350 μm sieve and the surviving invertebrates were counted, and the functional parameters detritus processing, bacterial activity and metabolic diversity, and development of Eh were determined. Detritus processing was measured as the concentration of dissolved organic matter in the overlying water visible as UV absorbance. To this purpose, the absorbance at 280 nm in the overlying water was measured at the end of the experiment.
We assessed bacterial activity and metabolic diversity in the sediment by community level physiological profiling (CLPP) using Biolog® GN microplates containing 95 unique single substrates (Biolog, Inc., Hayward, USA) (Garland and Mills, 1991). At the end of the experiment, pore water was sampled by pipetting 1 mL of sediment top layer, while preventing sampling of the overlying water. Samples were subsequently diluted 50x with DSW, and distributed over the 96 Biolog® GN wells. Plates were incubated for 48h and utilization patterns of 95 different single carbon sources were used to calculate the bacterial activity (average metabolic response, AMR) and community metabolic diversity (CMD) community (Garland, 1997).

We measured effects of copper on invertebrate locomotion and bioturbation as vertical sediment profiles of Eh recorded over time as described previously (Hunting et al., 2012). 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 [0–9-mm] depth, 2-mm width, every 15 min) throughout the experiment. Before the start of the actual experiment, 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.

Chemical analysis

Actual copper concentrations in the sediment at the end of the experiment were determined by digesting duplicate 130 mg oven-dried subsamples per treatment in 2mL of a 4:1 mixture of nitric acid (65% p.a.; Sigma-Aldrich) and hydrochloric acid (37% p.a., Sigma-Aldrich) in tightly closed Teflon bombs upon heating in an oven at 140 °C for 7 h. The digested samples were diluted with 8 mL of deionized water and allowed to settle overnight at 5°C. Copper concentrations in the samples were determined by flame atomic absorption spectrophotometry (Perkin-Elmer AAnalyst 100, Germany). The certified reference material ISE 989 Riverclay (Wageningen Agricultural University, The Netherlands) was used for quality assurance. The measured metal test concentrations were corrected for recovery and used to calculate the actual copper concentrations in the sediment per treatment.
Data analysis

The LC50, i.e. the actual toxicant concentration in the sediment at which 50% mortality was observed compared to the control, was calculated according to the logistic response model adopted from Haanstra et al. The following equation, \( y = \frac{c}{1+e^{b(\log(x) - \log(a))}} \), was fitted through the concentration response data, in which \( y \) represents the effect parameter (survival); \( x \) the actual exposure concentration; \( a \) the EC\(_{50}\); \( b \) the slope of the logistic curve; and \( c \) the average survival in the control.

To evaluate the impact of copper on the invertebrate-mediated functional parameters, we considered the produced DOC, and the AMR and CMD in the presence of invertebrates as relative to the control treatment without invertebrates, i.e. measurements in the control treatment were subtracted from the measurements in the invertebrate treatments (residuals). The EC\(_{50}\), i.e. the actual copper concentration in the sediment at which 50% reduction of the functional parameters was observed compared to the control without copper, was calculated as described above.

To evaluate the impact of copper on invertebrates locomotion and bioturbation, we considered the increase of sediment redox potential in the presence of invertebrates as relative to the control treatment without invertebrates, i.e. measurements in the control treatment were subtracted from the measurements in the invertebrate treatments (residuals). Eh residuals at day 5 of the experiment were subsequently plotted over depth against the actual copper concentrations by linear interpolation (Hammer et al., 2001).

Results

The actual copper concentrations in the sediment were: 5.6 (control), 15.3, 21, 55.9, 105.9, 176.2 and 487.6 mg Cu/kg dw sediment. This ranged between 88-111% of the nominal values in the 20-500 mg Cu/kg dw sediment range, but was higher than the nominal values of the 0 and 10 mg Cu/kg dw sediment microcosms. This is conform expectation as copper is an essential element.

Considering the results of the treatments without invertebrates, DOC, bacterial activity (AMR) and metabolic diversity (CMD) showed a gradual decrease with increasing copper concentrations in the sediment (Fig. S1), while Eh remained unaffected (Fig. S2). Results of the treatments containing the invertebrates Tubifex spp. and A. aquaticus are shown in figure 1a-h. All invertebrates in the control treatment without copper were still alive at the end of the experiment. Clear concentration response curves were observed for the effect of copper on invertebrate survival, and the functional parameters DOC, AMR and CMD. From these concentration-response curves, the actual EC\(_{50}\) values with their 95% confidence intervals
Fig. 1: Concentration-response curves for the effect of copper (Cu) in the sediment after the 5-day experiment on (A,E) survival (% of control); and the residuals [[treatment measurements] - [measurements in controls without invertebrates]] of (B,F) DOC concentrations in the overlying water; (C,G) Bacterial Community Metabolic Diversity (CMD); and (D,H) Average Metabolic Response (AMR) of the bacterial community. Provided are LC₅₀/EC₅₀ with 95% C.L.
Concentration-response curves of the functional parameters DOC, AMR and CMD in the presence of Tubifex spp. are similar to the concentration-response curve of Tubifex spp. survival and resulted in comparable EC_{50} values (Fig. 1 a-d). In contrast, the EC_{50} values of the functional parameters DOC, AMR and CMD in the presence of A. aquaticus are significantly lower than the EC_{50} of A. aquaticus survival (Fig. 1 e-h). Development of sediment Eh profiles over time differed between microcosms with and without invertebrates and between invertebrate treatments. Eh residuals at day 5 (invertebrate treatment minus control treatment without invertebrates) are presented in figure 2a,b. Tubifex spp. mainly enhanced Eh at the top layer of the sediment and in deeper layers of the sediment only at lower copper levels (Fig. 2a). This positive effect rapidly decreased with increasing copper concentrations, although a slight increase in Eh was visible in deeper layers (8-9 mm depth). Since no Tubifex spp. survived these copper levels, this observed enhancement of Eh is probably the result of continuous irrigation of the created burrow-network. In the absence of copper, A. aquaticus homogenized the entire top layer (1 cm) of the sediment, but this positive effect on Eh rapidly
decreased with increasing copper concentrations leaving the sediment completely stratified (Fig. 2b).

Discussion

The effect of toxic pressure on biotic interactions and ecosystem functioning remains poorly understood. Only a limited number of studies considered the effects of polluted leaf litter on invertebrates consumption (Brooks et al., 2009; Bundschuh et al., 2012), but the effect of toxicants on invertebrate-sediment interactions remains largely unknown. This study compared invertebrate survival and invertebrate mediated functional responses to sediment copper amendment and demonstrated that copper exposure resulted in reduced invertebrate bioturbation activities, microbial diversity and activity, and detritus processing, thereby illustrating that copper contamination significantly affects the link between invertebrate bioturbation and ecosystem functioning.

Bacterial metabolic diversity and metabolic activity also decreased with increasing copper concentrations in the absence of invertebrates. Bacterial conditioning of detritus is known to enhance invertebrate feeding of detritus (e.g. Graça et al. 2001, Canhoto and Graça 2008). It is thus possible that reduced decomposition was the result of reduced detrital palatability to invertebrates due to copper toxicity to the bacterial community, although bacterial conditioning typically becomes effective long after our experimental period (5-days) (e.g. Danger et al, 2012). Moreover, increasing evidence suggests that contaminated detritus has stronger impacts on invertebrates than on bacteria, and adversely affect invertebrate performances (Gonçalves et al. 2011). This is confirmed for a number of invertebrate species, showing that changes in feeding behavior is responsible for reduced decomposition rates (Macedo-Sousa et al. 2007, 2008, Pestana et al. 2007, Roussel et al. 2008). In the present study, the adverse effects of copper on decomposition thus relied on reduced invertebrate activity.

Invertebrate bioturbation typically enhances bacterial activity, detritus processing and sediment Eh (Solan et al., 2004; Mermillod-Blondin, 2011; Hunting et al., 2012), as observed in the present study. However, this positive effect decreased with increasing copper concentrations in the sediment. For *Tubifex* spp., the decrease in functional parameters coincided with the decrease in *Tubifex* spp. survival. In contrast, the decrease in functional parameters for *A. aquaticus* occurred at lower copper concentrations than the decrease in *A. aquaticus* survival. This is reflected by the sediment spatio-temporal redox profiles of the upper 10 mm of the sediment that visualized the interaction of invertebrates with the sediment. Bioturbation activities of *Tubifex* spp. and the dependent functional parameters clearly relied on the survival of *Tubifex* spp. In
contrast, *A. aquaticus* revealed a decreased influence on sediment Eh while the invertebrates were still alive and concomitantly a reduced influence of the invertebrate on ecosystem functioning. This suggests that sub-lethal copper concentrations reduced the bioturbating activities of *A. aquaticus*, resulting in a decreased decomposition, resulting in a decoupling of invertebrate community structure and ecosystem functioning.

The observed differences between *Tubifex* spp. and *A. aquaticus* in the effect of copper on the link between invertebrate survival and functional parameters between were likely caused by differences in locomotion and feeding behavior, i.e. *Tubifex* spp. lives within the sediment, while *A. aquaticus* is crawling on top of the sediment. Since copper was spiked to the sediment, *A. aquaticus* was able to avoid direct exposure to toxicants, while *Tubifex* spp. typically remains within the sediment and was therefore continuously exposed to toxicants. Avoidance behavior has often been observed for *A. aquaticus* when exposed to a variety of toxicants (e.g. Blockwell et al., 1997; Bundschuh et., 2011). Although a number of physiological and behavioral responses to toxicants (including reduced locomotion) have been observed for oligocheates (e.g. O’Gara et al., 2004), these did not impact the *Tubifex* spp.-sediment interaction in this study. This study therefore demonstrates that the sensitivity of functional parameters depends on the feeding strategy and locomotion of the invertebrate species.

This study demonstrated that toxicants can affect the functional links between invertebrate bioturbation and ecosystem functioning, highlighting the importance of species specific feeding strategies and locomotion for the effects of chemical stressors on ecosystem functioning. Here we show a decoupling of invertebrate community structure and ecosystem functioning under chemical pressure, suggesting that functional parameters (e.g. decomposition) may serve as more sensitive and reliable parameters for assessing ecological water quality and ecosystem functioning.

\[1\textit{Supplementary data related to this chapter can be found at: http://dx.doi.org/10.1016/j.envpol.2013.05.027.}\]
Chapter 4

DECOTAB – a multipurpose standard substrate to assess litter quality effects on microbial decomposition and invertebrate consumption

Published as:
Abstract: Currently available tools for studying plant litter decomposition and invertebrate consumption in aquatic ecosystems have at least 2 major limitations: 1) the difficulty of manipulating litter chemical composition to provide mechanistic insights into attributes of litter quality controlling decomposition rate, and 2) lack of a standardized litter that hampers comparisons of results among studies. These limitations point to a need for a standard litter surrogate with adjustable chemical composition. We propose using a decomposition and consumption tablet (DECOTAB) consisting of cellulose powder embedded in an agar matrix to evaluate decomposition and consumption rates in aquatic environments. We describe the preparation of DECOTABs and demonstrate some applications in laboratory microcosms and outdoor mesocosms. A leaf shredder, the isopod Asellus aquaticus, and a collector-gatherer, the nonbiting midge larva Chironomus riparius, readily consumed DECOTABs, leading to massive mass loss of the tablets within 21 d (~90%). The isopod also consumed DECOTABs amended with extracts of riparian plants and soil to create a chemically complex source of organic matter. Our results highlight the potential utility of DECOTABs to assess invertebrate contributions to organic matter decomposition in aquatic systems. In the absence of invertebrates, exposure of basic and complex DECOTABs to microorganisms resulted in significant mass loss within 21 d (10–25%), and addition of an antibiotic and fungicide suppressed microbial decomposition, suggesting that the tablets are useful for studying microbial processes. Complex tablets decomposed faster than the basic tablets, a result illustrating the importance of chemical composition of organic material for microbial decomposers. DECOTABs are a novel, versatile tool for addressing long-standing questions in aquatic ecology and environmental assessment.

Key words: litter decomposition, cellulose degradation, microorganisms, detritivores, benthos, functional ecosystem assessment, aquatic ecosystem health, methodology, standardization.
Decomposition of plant litter is a vital ecosystem process driven by both microorganisms and detritivores (Webster and Benfield 1986, Graça 2001, Gessner et al. 2010, Tank et al. 2010). The standard approach to studying decomposition in the field is to measure leaf mass loss from litter bags made of coarse or fine mesh that controls access of invertebrates differing in body size (Boulton and Boon 1991). Although not perfect, partly because of risks of hypoxia in fine mesh bags or litter fragmentation by turbulent flow in coarse mesh bags (Boulton and Boon 1991). This approach adequately mimics natural conditions in many situations and represents the influences of both litter quality and environmental factors on decomposition dynamics. Litter bags are the method of choice in comparative field studies (comparisons of litter types, locations, etc.) and often yield realistic estimates of local decomposition rates (Webster and Benfield 1986, Boulton and Boon 1991). Laboratory studies in microbial microcosms (Suberkropp 1991, Dang et al. 2009) and feeding trials with detritivores (Cameron and La Point 1978, González and Graça 2003) complement the litterbag approach by providing important insight into the decomposition process. However, rigorous tests of the mechanisms responsible for differences in decomposition are difficult with approaches relying on natural litter that varies in many quality attributes.

Standardized ways to measure decomposition rates that exclude confounding effects of varying litter quality are required to allow comparisons on large geographical and temporal scales, e.g., for studies on wide-ranging impacts of human activities (pollution, habitat modification, etc.) on ecosystem functioning in natural environments (Gessner and Chauvet 2002, Young and Collier 2009, Woodward et al. 2012). Selecting individual plant litter species for large-scale studies (Boyero et al. 2011) is only a partial remedy because litter quality can vary widely even within a given plant species (Lecerf and Chauvet 2008). Filter paper and cotton strips have long been used as substitutes for natural plant litter (Eggl 1964, Hildrew et al. 1984, Tiegs et al. 2007, Imberger et al. 2010). Cotton strips, in particular, are used as a standardized substrate for this purpose (e.g., Boulton and Quinn 2000, Young et al. 2008, Young and Collier 2009) because they are essentially composed of cellulose, a major component of plant litter, and have much greater tensile strength than filter paper.

Despite their potential utility in decomposition studies, cotton strips have limitations (Imberger et al. 2010). First, the previously used standard material (Shirley Soil Burial Test Fabric) is no longer available, so comparisons between present-day studies and earlier studies are complicated, although results with Shirley Soil Burial Fabric and replacement materials, such as ‘calico’ are correlated (Imberger et al. 2010). However, calicoes are produced from natural cotton and, therefore, have an uncertain and variable chemical composition. Second, the
The chemical composition of cotton strips is extremely simple (cellulose content > 95%) compared to natural plant litter, such that numerous leaf constituents (N, lignin, tannins, fatty acids, etc.) that are potentially important factors in decomposer activity and decomposition rate are lacking. Third, the composition of cotton strips is difficult to manipulate experimentally to test for effects of specific chemical plant constituents or of compounds inhibiting microbial or detritivore activity (e.g., antibiotics, fungicides, insecticides; see Rader et al. 1994). These limitations highlight the need to identify a standardized substrate whose composition can be altered according to the needs defined by the question posed.

The objective of our study was to develop and test a standardized proxy material whose chemical composition could be adjusted easily for experimental purposes. Inclusion of specific plant constituents and other compounds at desired concentrations in polycarbohydrate gels (e.g., agar or phytagel) has been useful in studies of allelochemical interactions (Hay et al. 1998), colonization dynamics of sessile invertebrates (Henrikson and Pawlik 1995, Hunting et al. 2010b), and feeding preferences of aquatic herbivores (Pavia and Toth 2000). We assessed whether this matrix could be modified to serve as surrogate plant material whose chemical composition can be controlled and whose texture broadly resembles that of natural litter. However, agar has a low nutritional value for invertebrates and can be degraded by only a limited number of microorganisms (Bärlocher and Porter 1986, Armisén 1991). Therefore, we developed and tested the performance of a decomposition and consumption tablet (DECOTAB) consisting of a high concentration of cellulose powder (75%) embedded in an agar matrix to evaluate the importance of factors affecting microbial decomposition and invertebrate feeding. We describe the preparation of the proposed DECOTABs and evaluate some potential applications in laboratory microcosms and outdoor mesocosms.

**Methods**

*Preparation of DECOTABs*

We made basic DECOTABs from a suspension containing 60 g of powdered cellulose (Sigma–Aldrich, St. Louis, Missouri), 20 g of purified agar (OXOID Ltd., Basingstoke Hampshire, UK), and 60 μmol ascorbic acid/L deionized water (dH₂O) as an antioxidant (Merck GmbH, Darmstadt, Germany) (Niki 1991). We heated the mixture to 100°C to dissolve the agar, allowed it to cool to 50°C with frequent stirring, and poured it into a multiwell polycarbonate mold (15-mm diameter, 5-mm height) to cast tablets with a final volume of 118 mm³ (Fig. 1A). The tablets initially had a convex surface that quickly flattened during solidification of the agar at 7°C. The tablets could be stored in closed containers for up to 3 wk in the
refrigerator without noticeable decay or dehydration. We measured the dry mass of freshly prepared tablets (mean ±1 SD = 81.3 ± 3.1 mg, \( n = 25 \)) by drying them for 3 d at 70°C and then weighing them with an analytical balance (precision = 0.1 mg; Mettler AT261, Mettler-Toledo, Tiel, The Netherlands).

We prepared DECOTABs including an antibiotic and fungicide as described above except that we added chloramphenicol (60 mg/L \( \text{dH}_2\text{O} \); Sigma–Aldrich) and cycloheximide (60 mg/L \( \text{dH}_2\text{O} \); Sigma-Aldrich) to the cooled suspension at 50°C. We prepared DECOTABs of more complex composition to enhance their resemblance to natural particulate organic matter (POM). These tablets had the same volume as the basic DECOTABs, but consisted of 46.7 g of cellulose, 20 g of purified agar, and 60 \( \mu \text{mol} \) of ascorbic acid/L \( \text{dH}_2\text{O} \). We extracted plant constituents from bulk standardized garden soil (Baseline, Maxeda DIY, Diemen, The Netherlands), stinging nettle (\textit{Urtica dioica}), and willow leaf extract (\textit{Salix alba}), both common plants in the riparian zone of temperate rivers in Europe. We used 70% acetone as the extraction solvent and air-dried the extract (Hunting et al. 2010a). We added powdered extracts (6.7 g/L for soil; 3.3 g/L for stinging nettle, and 3.3 g/L for willow leaves) to the cooled suspension at 50°C.

\[\text{Fig. 1. Polycarbonate mold used to prepare DECOTABs (A), and isopods (Asellus aquaticus) rallying around and feeding on a basic cellulose DECOTAB (B).}\]
**Experiment 1: Microbial vs invertebrate-mediated decomposition**

We ran an experiment in laboratory microcosms to evaluate the relative contribution of microorganisms and detritivores to decomposition of DECOTABs. We used 100-mL glass microcosms with sediment containing 50 g quartz sand (0.1–0.5-mm grain size; Dorsilit, Eurogrit, Papendrecht, The Netherlands) and a mixture of standard culture food (7.5 mg) composed of Trouvit® (Trouw, Fontaine-les-Vervins, France) and Tetraphyl® (Tetrawerke, Melle, Germany) at a ratio 20:1 as organic material. The overlying water consisted of 250 mL Dutch Standard Water (Maas et al. 2002). To obtain a natural microbial inoculum, we added 50 mL of filtered (<75 µm) natural surface water from a local shallow lake that typically supports a diverse microbial community (del Giorgio and Gasol 1995). A detailed description of Dutch freshwater bacteria in comparable lakes was published by Zwart et al. (1998, 2002). We aerated the water gently with compressed air throughout the experiment.

We tested 2 invertebrate species: the nonbiting midge larva *Chironomus riparius* (laboratory culture) and the isopod *Asellus aquaticus* (collected from a nearby shallow lake). We used relationships among invertebrate length, fresh mass, and dry mass to standardize invertebrate biomass (Hunting et al. 2012). We added invertebrates based on equal initial dry mass. Thus, we added either 5 *A. aquaticus* (7–9 mm) or 20 *C. riparius* larvae (2nd instar) to each microcosm and placed 1 DECOTAB in each microcosm. Treatments were: 1) basic DECOTAB, no invertebrates; 2) DECOTAB containing an antibiotic and fungicide, no invertebrates; 3) basic DECOTAB and *C. riparius*; and 4) basic DECOTAB and *A. aquaticus*. We did not include a treatment with DECOTAB containing both antibiotics and invertebrates because of the toxicity of both antibiotics to invertebrates (Baliga et al. 1970, Monari et al. 2008). We replicated each treatment 10 times. After 21 d, we removed the DECOTABs with a needle, rinsed and dried (3 d at 70°C) them, and weighed them with an analytical balance (precision = 0.1 mg; Mettler AT261). We subtracted the final dry mass from the estimated initial dry mass to calculate DECOTAB mass loss. We tested for treatment differences with a 1-way nonparametric permutation-based multivariate analysis of variance (PERMANOVA) based on Bray–Curtis distances and 9999 permutations (Anderson 2001), followed by a Bonferroni-corrected PERMANOVA pairwise comparisons in PAST (Hammer et al. 2001).

**Experiment 2: Effects of DECOTAB complexity on decomposition and consumption**

We ran a 21-d mesocosm experiment in June 2011 to evaluate the performance of DECOTABs under outdoor conditions and to compare basic and complex DECOTABs containing organic matter extracted from plants.
and soil. Mesocosms consisted of rectangular (66 cm long × 34 cm wide × 30 cm high) 90-L plastic tubs. They contained ~40 L of rainwater and 18.5 L of sediment made of standardized garden soil (Baseline) and quartz sand (0.1–0.5 mm; Dorsilit, Eurogrit, Papendrecht, The Netherlands) mixed in a ratio of 5 L soil/25 kg sand. We placed the mesocosms in concrete containers filled with water to buffer temperature fluctuations. We pulled a gauze screen (mesh size = 1 mm) over the concrete containers to reduce colonization by allochthonous fauna. Before starting the experiment, we allowed the mesocosms to sit for 2 d to allow the sediment to settle. We assumed that microbial communities could sufficiently acclimate during these 2 d. Subsequently, each mesocosm received 3 basic and 3 complex DECOTABs and either no invertebrates or A. aquaticus. We added the isopods at densities of 402 individuals (ind.)/m² (90 ind./mesocosm), which falls within the density range reported for natural populations (67–586 ind./m²; Adcock 1979). We replicated both treatments 5 times. During the experiment, we gently aerated the overlaying water with a permanently installed air compressor aeration system. After 21 d, we removed the DECOTABs and weighed them as described above. We tested for treatment differences with a 2-way factorial PERMANOVA based on Bray–Curtis distances and 9999 permutations (Anderson 2001), followed by a Bonferroni-corrected PERMANOVA pairwise comparisons in PAST.

Fig. 2. Mean (±1 SD) mass loss of cellulose DECOTABs in laboratory microcosms stocked with 1 of 2 invertebrate species (Asellus aquaticus or Chironomus riparius) or inoculated with microorganisms (t = 21 d). Bars with different letters are significantly different (Bonferroni-corrected permutation-based multivariate analysis of variance [PERMANOVA] pairwise comparisons.)
Results

DECOTAB mass loss differed between treatments in the microcosm experiment ($F = 23.61, p < 0.0001$; Fig. 2). Both *C. riparius* and *A. aquaticus* fed actively on the basic DECOTABs (Fig. 1B), resulting in 80 to 90% mass loss over the course of the 21-d experiment. In microcosms without invertebrates, mass loss averaged 10 to 25% of the initial mass, and no mass loss occurred when DECOTABs contained an antibiotic and fungicide (Fig. 2).

Mass loss in outdoor mesocosms was higher in the presence of invertebrates than in the microorganism-only treatment ($F = 317.4, p = 0.0001$; Fig. 3). Mass loss of complex DECOTABs containing soil and plant extracts was higher than that of basic DECOTABs when only microorganisms were present ($F = 13.0, p = 0.002$; pairwise comparison, $p = 0.031$), but this difference was not apparent when isopods were allowed to feed on DECOTABs (pairwise comparison, $p = 0.43$). Mass loss in the presence of invertebrates was similar in outdoor mesocosms and laboratory microcosms in the presence of *A. aquaticus* (cf. Figs 2, 3).

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*Fig. 3. Mean (±1 SD) mass loss of basic cellulose DECOTABs and complex DECOTABs containing plant and soil extracts mediated by *Asellus aquaticus* or microorganisms in outdoor mesocosms (t = 21 d). Bars with different letters are significantly different (Bonferroni-corrected permutation-based multivariate analysis of variance [PERMANOVA] pairwise comparisons.*
Discussion

The new standardized plant litter substitute developed and tested in our study was useful for measuring rates of microbial decomposition and consumption by invertebrates in sediments. Both invertebrate test species fed actively on the DECOTABs, resulting in a dramatic mass loss, despite the presence of other sources of organic matter in the sediments of our micro- and mesocosms. Moreover, DECOTABs exposed to microorganisms in the absence of invertebrates also lost mass in the 2 experiments, whereas mass loss was suppressed in microcosms with DECOTABs containing an antibiotic and a fungicide. These results suggest that DECOTABs are a useful substrate for studying both microbial decomposition and consumption of organic matter by invertebrates in aquatic systems. However, the extent to which they reflect decomposition of natural plant litter and could be used to develop functional metrics for assessing impacts of anthropogenic stressors on aquatic ecosystems remains to be tested.

DECOTABs were consumed by a shredder (A. aquaticus) and by C. riparius, which burrows in sediments and feeds on fine particulate organic matter (Cummins and Klug 1979). Chironomids readily consumed DECOTABs, a result suggesting that DECOTABs could be used to study mechanisms affecting use of organic matter by collector-gatherers (in addition to shredders) and to measure their role in organic-matter dynamics of aquatic ecosystems.

Experimental manipulation of substrate composition facilitates mechanistic insights into decomposition processes. One could test whether and which lipids are triggers for detritivory, growth, and reproduction (Cargill et al. 1985). One could assess whether particular phenolics or mixtures of phenolics affect microbial activity or detritivore performance in laboratory or field conditions. We supplemented cellulose DECOTABs with an antibiotic and fungicide to assess whether and to what extent microorganisms vs detritivores contribute to decomposition.

Investigators have used various approaches to assess the contribution of shredders to litter decomposition (Petersen and Cummins 1974, Cuffney et al. 1990, Hieber et al. 2002, González and Graça 2003), but the available information is still scarce (Boulton and Quinn 2000, Young et al. 2008). We did not compare DECOTABs with natural plant litter, so our results are not directly comparable with estimates based on measured litter consumption (Hieber and Gessner 2002, Hunting et al. 2012). Nevertheless, our data corroborate the notion that invertebrates can play dominant roles in organic-matter turnover in aquatic ecosystems (Wallace and Webster 1996). Invertebrates contributed up to 55 to 65% of total DECOTAB mass loss, whereas microorganisms contributed only 10 to 25%.

Plant litter is a complex mixture of structurally diverse compounds. To
mimic the composition of natural litter in mesocosms, we created DECOTABs containing extracts of riparian plants and soil and offered them to microorganisms only or to microorganisms in combination with A. aquaticus. The differential response of microbes to basic cellulose and complex DECOTABs illustrates the importance of resource composition for microbial decomposers. For invertebrates, a similar distinction was not observed, possibly because resemblance in texture masked differences in chemical composition. However, conclusive answers about the relative importance of texture vs chemistry for DECOTAB palatability require tests with tablets differing more widely in chemical composition.

Standardized measures to determine decomposition rates are increasingly important for assessing effects of anthropogenic stressors on ecosystem processes (Gessner and Chauvet 2002). However, methods differ among studies, and some are based on using litter from different plant species that decompose at different rates. Even litter of the same species may vary in chemical composition and texture if collected at different locations (Lecerf and Chauvet 2008) or at different times. This variability hampers comparisons of decomposition dynamics over large spatial and temporal scales (Boyero et al. 2011). Use of a standardized substrate would facilitate comparisons among studies, including studies aimed at assessing the response of ecosystem functioning to anthropogenic stressors. The DECOTAB approach could facilitate such standardized experiments at larger scales and increase power of meta-analyses.

In conclusion, our study demonstrated the potential of the newly developed DECOTABs for a variety of applications in aquatic environments. The DECOTABs described here were based on an aqueous matrix, so they shrink upon dehydration. Therefore, they cannot be used readily in terrestrial systems because dehydration will affect shape and texture. However, the basic concept could be applicable to terrestrial studies if tablets were prepared dry. One of the greatest assets of DECOTABs is that they can be prepared in almost any desired size, shape, or composition to suit the needs of the specific question to be examined. Thus, they have the potential to become a highly standardized and versatile tool to address long-standing issues in aquatic ecology and environmental assessment.
Chapter 5

Solar radiation shapes bacterial functional diversity in sediments

Manuscript under review:
Abstract: Solar radiation is known to influence the species composition of bacterial communities. UV radiation can directly affect bacteria or alter the composition of organic matter, rendering available substrates for bacteria. However, effects of solar radiation on bacterial communities residing in sediments remain completely unexplored. This study investigated the influence of mimicked solar radiation on bacterial functional diversity in laboratory sediments. Two different organic matter sources, labile and recalcitrant organic matter (OM), were used and metabolic diversity was measured with Biolog GN. Radiation exerted strong negative effects on the metabolic diversity in the treatments containing recalcitrant OM, more than in treatments containing labile OM. The functional composition differed significantly between the treatments. Our findings demonstrate that a combined effect of light and OM shapes the functional composition of microbial communities developing in sediments, and acts as an important sorting mechanism for bacterial communities in wetlands.

Keywords: Organic matter quality, Solar radiation, benthic bacterial communities, bacterial metabolic diversity
Several studies have demonstrated that solar radiation may affect bacterial communities (e.g. Baldy et al. 2002; Piccini et al. 2009; Zepp et al., 2011). Especially UV radiation exerts negative effects on bacterial communities due to its detrimental effects on DNA (e.g. Santos et al. 2012a), but solar radiation may also change the chemical composition and palatability of organic compounds by photodegradation (e.g. Engelhaupt et al. 2002; Sulzberger and Durisch-Kaiser, 2009). Such changes in the chemical composition of OM may subsequently cascade towards shifts in bacterial community composition due to the interplay between bacterial resource niches (i.e. the type of substrates that are utilized) and available resources (e.g. Salles et al. 2009). This suggests that the functional composition of bacterial communities may also change when exposed to solar radiation.

Several studies have demonstrated that photolytic changes of OM can result in enhanced bacterial production (e.g. Wetzel et al., 1995), and Santos et al. (2012b) indeed provided some evidence that UV-B radiation can induce shifts in the functional composition of bacterioplankton communities. However, potential effects of solar radiation on bacterial communities residing in sediments remain completely unexplored. This study therefore aimed to investigate the effect of solar radiation on the functional composition of bacterial communities in shallow aquatic sediments, and compared the effect of light and dark incubation on bacterial metabolic diversity in sediment microcosms. Since composition of the available OM is one of the main drivers of bacterial community composition (e.g. Hättenschwiler and Vitousek 2000; Myers et al. 2001; Baldy et al., 2002; Docherty et al., 2006), we assessed the effects of light exposure on bacterial communities inhabiting either a labile or a recalcitrant OM source.

**Methods**

*Sediment microcosms*

Freshly collected stinging nettle, *Urtica dioica*, was used as a labile OM source, and intact peat collected from natural peatlands was used as recalcitrant OM source. Both nettle and peat were frozen in liquid nitrogen and thoroughly ground in a pestle and mortar. Quartz sand (0.1-0.5 mm; Dorsilit, Eurogrit, Papendrecht, The Netherlands) was mixed with either the labile or recalcitrant OM source (95:5 weight ratio sand:OM-source with final dry weight OM concentrations of 0.63% and 0.52% for labile and recalcitrant OM, respectively), and then autoclaved. 5 mL of sediment was subsequently added to each of the 5 replicate microcosms per treatment (plastic round vials (Greiner Bio-one, Germany): 27 mm diameter, 5 cm height), resulting in ~1 cm sediment layer. Each microcosm received 2 cm of overlying water (Dutch Standard Water, DSW; deionized water with 200
mg/L CaCl$_2$·2H$_2$O, 180 mg/L MgSO$_4$·H$_2$O, 100 mg/L NaHCO$_3$ and 20 mg/L KHCO$_3$; pH = 8.2 ± 0.2). A mixture of sediment pore-water and surface water collected from 2 different natural wetland systems was added as bacterial inoculum.

**Experimental set up**

To mimic natural conditions and exposure to solar radiation, we incubated the samples at 15°C under a dark:light regime of 12h:12h. We used mercury lamps (Arcadia-D3, Redhill, United Kingdom: 160W; luminous flux 1900 lm) that, in addition to emission of visible light (400-800nm), emit UV radiation (UV-B 1.75 W.m$^{-2}$ at 310 nm; UV-A 10 W.m$^{-2}$ at 365 nm). These intensities of UV-radiation are commonly registered in temperate areas (Kelly et al., 2003). The duration of the incubation was 5 days. An additional set (n=5) of microcosms of both OM types was incubated in the dark as control. This yielded a total of four treatments, consisting of: 1) labile OM in light; 2) labile OM in the dark; 3) recalcitrant OM in light; and 4) recalcitrant OM in the dark. Each treatment was replicated 5 times. After 5 days, bacterial metabolic diversity was determined as described below.

**Community metabolic diversity (CMD)**

Community metabolic diversity (CMD) in the sediment was assessed by community level physiological profiling (CLPP) using Biolog® GN microplates containing 95 unique single substrates (Biolog, Inc., Hayward, USA; Garland and Mills 1991). Biolog GN plates are comprised of simple, common substrates (e.g. Sucrose, Mallose and Citric Acid), and do not include recalcitrant substrates nor specific substrates typical of the OM used in this study. It is therefore impossible to directly relate substrate utilization profiles to the actual functioning of the developed bacterial communities. Nonetheless, the number of substrates used can serve as a proxy of the metabolic diversity of the bacterial community, and differences in utilization profiles indicate that functionally distinct bacterial communities can develop depending on treatment (Garland 1999). CMD was determined after 5 days of incubation. Pore water was sampled by pipetting 1 mL of the sediment top layer, while preventing sampling of the overlying water. Samples were subsequently diluted 30x with DSW and distributed over the 96 Biolog® GN wells. Plates were incubated for 36h at 37°C and utilization patterns of 95 different single carbon sources were measured at 490 nm using an automated microplate reader (VERSAmax tunable microplate reader, Molecular Devices, Sunnydale, USA). This data was used to calculate the CMD (Garland 1997) using a threshold absorbance of 0.15, and analyzed with a two-way ANOVA and Tukey-HSD post hoc test. To relate the bacterial functional composition to the four
treatments, utilization patterns of the 95 carbon sources were analyzed using a Bray-Curtis-based cluster analysis and a two-way ANOSIM (Hammer et al. 2001).

Results

Effects of light on the metabolic diversity of the different treatments are presented in Figure 1. No significant difference was observed between the light exposure and the control dark incubation in the sediments containing labile organic matter. In contrast, light exposure significantly reduced the community metabolic diversity (CMD) in the treatments containing recalcitrant DOM (two-way ANOVA, Tukey HSD, p=0.004; Fig. 1).

In addition to the number of substrates used by the bacterial community, we assessed which set of substrates was used to compare the functional composition of the communities that developed during the incubation. A two-way Analysis of Similarity (two-way ANOSIM) results revealed that the bacterial resource niches differed significantly between treatments depending on both radiation and organic matter type (two-way ANOSIM: Light R=0.536, p=0.0007; OM R=0.302, p=0.0146, respectively) (Fig. 2), showing that the developed bacterial communities were functionally distinct.

![Figure 1](image)

*Fig. 1: Mean (± S.E.) community metabolic diversity (CMD) of the four treatments after five days incubation. Bars with the same letters are not significantly different (two-way ANOVA with Tukey's HSD post hoc test, n=5, p<0.05).*
Radiation diminished the number of organic substrates used and reduced the similarity of substrate use between bacterial communities. This was most evident when peat was used as substrate, and this might have been provoked by either visible light and/or UV. The potential detrimental effects of UV radiation on e.g. DNA and enzymes (e.g. Santos et al. 2012a) are typically held responsible for this negative effect, but we speculate that compounds liberated during radiation mediated degradation of recalcitrant OM negatively affected some members of the bacterial community. It has been demonstrated that photo-degradation of OM creates useful low molecular weight compounds, as well as toxic hydrogen peroxide and various free radicals (e.g. Mopper and Zhou, 1990; Scully et al., 1996). Substituted organic molecules and aromatic products may also from during this process (Mill et al., 1980). Recalcitrant OM, in contrast to labile OM, contains substantial amounts of aromatic compounds (e.g. humic acid) that are known to strongly absorb UV-B (Zepp

Fig. 2: Bray-Curtis-based dendrogram representing level of similarity between sets of substrates used by the bacterial communities after five days incubation at four different conditions.
et al., 1985). Solar radiation is composed of visible light and UV-radiation, and therefore more likely to affect the chemical composition of recalcitrant OM than labile OM, explaining why in the present study radiation effects were most prominent on peat. Thus, although the importance of these indirect effects of photo-degradation or photo-activation for bacterial community structure and productivity cannot be extrapolated to natural substrates and natural radiation, our data support the notion that the effect of solar radiation differs depending on the structural composition of the organic matter (cf. Tranvik and Kokolj 1998; Engelhaupt et al., 2002; Docherty et al., 2006; Köhler et al., 2012). More importantly, the present study showed that the interaction of bacterial community metabolism with radiation and recalcitrance of organic substrates may occur at the boundary of sediment and water, a prominent habitat in mudflats and wetlands. This suggests that both solar radiation and OM composition are important drivers shaping bacterial communities in shallow benthic environments, demonstrating that solar radiation is a currently overlooked, but important sorting mechanism (Mann and Wetzel 1995; Santos et al. 2012b) in wetlands.

In conclusion, solar radiation alters and diminishes the metabolic diversity of bacterial communities in peat containing sediments, and that solar radiation shapes the functional composition of bacterial communities in shallow wetland sediments.
Chapter 6

Invertebrates as driver for decomposition, sediment mixing and bacterial communities: An outdoor mesocosm experiment

Manuscript under review.
Abstract: Decomposition of organic matter is a central ecosystem process governed by microorganisms and invertebrates. Several parameters are at hand to quantify the decomposition process, but these are seldom studied in coherence and mainly rely on laboratory experiments. Therefore the aim of this study was to test the response of bacterial functional diversity and activity, biogenic mixing depth and detritus processing to activities of invertebrate species combinations in outdoor mesocosms. Metabolic diversity and activity of the bacterial communities did not differ between treatments, indicating that invertebrates did not influence bacterial activity. The biogenic mixing depth (BMD), represented by the depth until the average redox potential is enhanced relative to the control, increased with increasing number of invertebrate bioturbator types, rather than with increasing number of species. Detritus processing, measured as DECOTAB mass loss, was substantially higher in treatments containing invertebrates compared to the control treatment without invertebrates. For DECOTAB mass loss, the presence of shredders (the isopod A. aquaticus or the amphipod G. pulex) was of predominant importance, demonstrating the importance of functional identity rather than diversity. By studying several functional parameters in coherence, we demonstrated that different ecosystem processes responded differently to invertebrate species composition. We showed that the sentinel for decomposition (DECOTAB mass loss) reflected the functional composition of the invertebrate community, whereas the microbial parameters did not, despite clear-cut effects on biogenic mixing. It was indicated that solar radiation and its consequent effects on the variable redox conditions may decouple invertebrate-bacterial interactions.

Key words: decomposition, aquatic invertebrates, bioturbation, functional traits, bacterial community structure, cellulose decomposition, redox potential.
Decomposition of organic matter is a central process in ecosystem functioning and therefore considered a promising proxy to evaluate the health of ecosystems (e.g. Gessner et al. 2010; Kampfraath et al. 2012). Detritus processing is driven by invertebrate and microbial activities, in which processing rates are the result of direct consumption and invertebrate bioturbation activities that promote microbial decomposition (Covich et al. 2004, Nogaro et al. 2009, Hunting et al. 2012).

The significance of invertebrate species composition for detritus processing is typically evaluated with a number of functional parameters. Firstly, various studies have demonstrated that invertebrate activities promote bacterial activities, the stimulus depending on the type of invertebrate sediment reworking (Mermillod-Blondin and Rosenberg 2006; Nogaro et al. 2009). In accordance, microbial community structure was observed to be affected by bioturbation (Bertics and Ziebs 2009; Hunting et al. 2012). However, metabolic diversity or substrate utilization patterns, may be a more important attribute of ecosystem functioning than bacterial community structure (Salles et al. 2009; Gravel et al. 2011). Secondly, invertebrates have been shown to affect the geochemical characteristics of the sediment (Meysman 2006). This is often quantified by following the redistribution of chemical tracers such as luminophores (e.g. Mermillod-Blondin et al. 2002), digital imaging of sediment cross-sections (e.g. Solan et al. 2004) and profiling oxygen penetration (e.g. Birchenough et al. 2012), or redox potential (Hunting and Van der Geest 2011; Hunting et al. 2012). Thirdly, standardized substrates such as cotton strips or DECOTABs that are composed mainly of cellulose, a major constituent of plant litter, have been used to obtain measurements of the cellulose decomposition potential as a standardized surrogate measure reflecting litter decomposition (Boulton and Quinn 2000; Tiegs et al. 2008; Young et al. 2008; Imberger et al. 2010; Kampfraath et al. 2012). These three groups of functional parameters are rarely studied simultaneously, especially not in experiments, and therefore the relative importance, reliability and cohesion remain uncertain. Moreover, most of our understanding of invertebrate interactions with sediments and their effects on decomposition is based on laboratory single species experiments.

Experiments are thus required that test the predictive potential of functional metrics in invertebrate detrital food webs under (quasi) natural conditions. Therefore, our study aimed to test whether a simultaneous response of a number of functional parameters to activities of multispecies invertebrate assemblages could be derived from their responses to single invertebrate species experiments under laboratory conditions. To this purpose, bacterial functional diversity and activity, sediment redox potential and DECOTAB mass loss were measured in laboratory microcosms and outdoor mesocosms in the presence of single invertebrate
species and manipulated multi-species assemblages.

**Methods**

*Mesocosms and test organisms*

**Mesocosms.**— A 21-d mesocosm experiment was performed in June 2011. Our outdoor mesocosms consisted of rectangular 90 L plastic tubs (L*W*H, 66 x 34 x 30 cm, respectively). They contained ca. 40 L of rainwater and 18.5 L of sediment made of standardized garden soil (Baseline, Maxeda DIY, Diemen, The Netherlands) and quartz sand (0.1-0.5 mm; Dorsilit, Eurogrit, Papendrecht, The Netherlands) mixed in a ratio of 5 L soil per 25 kg sand. These mesocosms were placed in concrete containers filled with water to buffer temperature fluctuations. A gauze screen (mesh size 1 mm) was pulled over the concrete containers to reduce colonization by allochthonous fauna. Before the experiment started, the mesocosms were left for two days to allow the sediment to settle and the microbial communities present in the soil and rainwater to acclimate. During the experiment, the overlaying water was gently aerated with a permanently installed air compressor aeration system. Sediment temperature and solar radiation (pyranometer connected to a datalogger, CR 10X) were measured every 5 min.

**Invertebrates.**—We used 5 invertebrate species that represented 3 types of bioturbators as outlined by e.g. Nogaro et al. (2009). The isopod *Asellus aquaticus* and the amphipod *Gammarus pulex* are omnivorous sediment-dwellers that act as biodiffusors, i.e., grazing the upper layer of detritus and biofilms on sediment particles. Larvae of the nonbiting 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 design**

Interactions between species were evaluated by considering the responses of functional parameters to multispecies assemblages as relative to their response to invertebrate single species incubations. To unravel potential interactions between invertebrate species and bioturbation type, the following invertebrate combinations were tested in outdoor mesocosms: [Tubifex spp. and *A. aquaticus*], [Tubifex spp., *A. aquaticus* and *C. riparius*], and [Tubifex spp., *A. aquaticus*, *C. riparius*, *L. variegatus* and *G. pulex*]. A control treatment without invertebrates was included and each treatment was replicated 5 times. Invertebrates were added up to equal total quantities of 500 mg.DW/m². After 21-d, we evaluated the influence of invertebrate species composition on detritus processing.
(DECOTAB mass loss), bacterial activity (AMR) and functional diversity (CMD), and Biogenic Mixing Depth (BMD) by monitoring the development of the sediment redox potential (Eh) as described below.

To evaluate whether responses of functional parameters to multispecies invertebrate assemblages can be predicted from single species incubation under laboratory conditions, single species were simultaneously incubated in the laboratory in order to obtain the responses of the selected functional parameters required to calculate the expected values in mixed species assemblages as described below. One representative of each bioturbation type (i.e., biodiffusor, upward conveyor, and gallery diffuser) was incubated for 3 weeks in 100 mL glass laboratory microcosms with the same substrate and invertebrate densities as applied in the outdoor mesocosms. Each treatments was replicates 5 times. The average responses in single species incubations were used to calculate an expected response to the mixed assemblages in the outdoor mesocosms as described below (Data analysis).

**Functional parameters**

**Bacterial activity (AMR) and functional (metabolic) diversity (CMD).**—Bacterial activity (AMR) and metabolic diversity (CMD) in the sediment was assessed by community level physiological profiling (CLPP) using Biolog® GN microplates containing 95 unique single substrates (Biolog, Inc., Hayward, USA) (Garland and Mills 1991). At the end of the experiment, pore water was sampled by pipetting 1 mL of sediment top layer, while preventing sampling of the overlying water. Samples were subsequently diluted 50x with Dutch Standard Water (DSW; a standardized synthetic analog of common Dutch surface waters, containing: 200 mg CaCl$_2$•2H$_2$O, 180 mg MgSO$_4$•7H$_2$O, 100 mg NaHCO$_3$, and 20 mg KHCO$_3$/L demineralized H$_2$O; pH 8.1, hardness 210 mg/L CaCO$_3$, alkalinity 1.2 meq/L), and distributed over the 96 Biolog® GN wells. Plates were incubated for 48h and utilization patterns of 95 different single carbon sources were used to calculate the bacterial activity (average metabolic response, AMR) and community metabolic diversity (CMD) community (Garland 1997).

**Biogenic Mixing Depth (BMD).**—Sediment redox potential (Eh) profiles were used to characterize invertebrate interactions within sediment as previously described (Hunting et al., 2012). We measured Eh in 3 replicates of all treatments. We recorded Eh with permanently installed redox potential microelectrodes (each mm [0–7-mm] depth, 2-mm width, every 15 min) and a calomel reference electrode connected to a Hypnos data logger (MVH consult, Leiden, the Netherlands) (Vorenhout et al. 2011). Biogenic mixing depth (BMD) *sensu* Solan et al. (2004) was subsequently determined by evaluating at which sediment depth an increase in the average Eh over time was visible as relative to the control mesocosms.
Fig. 1. Responses of functional parameters (mean ± SD) to multispecies invertebrate assemblages compared to controls without invertebrates: (A) bacterial Community Metabolic Diversity (CMD); (B) Average Metabolic Response (AMR); (C) Biogenic Mixing Depth (BMD); (D) DECOTAB mass loss. Different letters indicate significant differences between treatments (one-way analysis of variance, Tukey’s Honestly Significant Difference post hoc test, n = 5, p < 0.05). Number of species and bioturbator types are listed.
without invertebrates.

**Detritus Processing.**—Detritus processing was measured as cellulose decomposition with cellulose-based decomposition and consumption tablets (DECOTABS) (Kampfraath et al. 2012). Each mesocosm received three DECOTABS (Ø 15 mm, h 5 mm, final volume 118 mm³). DECOTABS were prepared from a mixture of 60 g cellulose (Sigma-Aldrich - #C6413), 20 g purified agar (OXOID Limited) and 60 μM ascorbic acid (Merck) per liter dH₂O. After 21 days, the DECOTABS were removed and subsequently rinsed, dried (three days at 70°C) and weighed. This weight was subtracted from the original dry weight to calculate the daily DECOTAB mass loss.

**Data analysis**

Utilization patterns of the 95 carbon sources were analyzed using a Jaccard-based cluster analysis and one-way ANOSIM with subsequent Bonferroni corrected pair-wise comparison (Hammer et al. 2001). Expected bioturbation type-specific (Biodiffuser, Gallery diffuser and Upward conveyor) values for the selected functional parameters were derived from single species incubations. The observed consumption rates (DECOTAB mass loss) and microbial AMR and CMD values for each invertebrate assemblage in the mesocosms were compared with the functional parameter values that would be expected if no interactive effects were present (cf. Chapman et al. 1988; Blair et al. 1990; Wardle et al. 1997). Expected values for mixed invertebrate assemblages were calculated based on bioturbation type as the weighted average of their contribution to the invertebrate assemblage at the start of the mesocosm experiment. The residuals (Observed - Expected) were tested against the null hypothesis (no interaction) that the average residual equaled 0 (one sample T-test) (Hammer et al. 2001).

**Results**

Responses of the functional parameters are presented in Fig. 1A-D. All treatments showed a similar range of Bacterial Community Metabolic Diversity (CMD) and Average Metabolic Response (AMR), including the control without invertebrates (Fig. 1A,B). There was a considerable variability between replicates, and therefore the influence of invertebrates on bacterial community diversity and activity could therefore not be assessed. Cluster analysis of the metabolic diversity of the bacterial communities of the different treatments did not reveal any separation between the different treatments and again showed considerable variability between replicates, including the control (Fig. 2) (ANOSIM, Bonferroni-corrected pair-wise comparison: p = 0.88), indicating that potential influences of invertebrate assemblages on the functional composition of the bacterial community could not be detected (Fig. 2). We
Fig. 2. Jaccard based cluster analysis representing the level of similarity of the bacterial Community Metabolic Diversity (CMD) of the control (C1-3), and triplicate invertebrate containing treatments, including combinations of Tubifex spp. (T), Asellus aquaticus (A), Chironomus riparius (C), Lumbriculus variegates (L), and Gammarus pulex (G). The functional composition did not differ between treatments (one-way ANOSIM, p = 0.878).
therefore did not compare expected and observed values for bacterial responses to the different invertebrate assemblages.

Spatiotemporal redox profiles revealed diurnal patterns that were coupled with solar radiation (Fig. 3), as Eh increased simultaneously with increasing solar intensity. The Biogenic Mixing Depth (BMD), represented by the depth until the average Eh is enhanced as relative to the control, differed depending on the combination of invertebrates (Fig. 2C), in which the invertebrate combinations [Tubifex spp., A. aquaticus, C. riparius] and [Tubifex spp., A. aquaticus, C. riparius, L. variegatus and G. pulex] revealed a significantly (One-Way ANOVA, Tukey-HSD, $p < 0.05$) enhanced BMD as compared to the invertebrate combination [Tubifex spp. and A. aquaticus]. The residuals (Observed values – Expected values derived from invertebrate monocultures, supplementary Fig. S1) revealed that no significant interaction was visible in the [Tubifex spp. and A. aquaticus] treatments (Fig 4A). In contrast, residuals were significantly higher than 0 in the invertebrate treatments [Tubifex spp., A. aquaticus and C. riparius] and [Tubifex spp., A. aquaticus, C. riparius, L. variegatus and G. pulex], suggesting a significant enhancement of biogenic mixing compared to the single species incubations.

Fig. 3. Examples of redox potential (Eh) profiles in depth (0-9 mm) and time (4 days) of the mesocosms containing A) no invertebrates; B) Tubifex spp. and Asellus aquaticus and C) Gammarus pulex, Asellus aquaticus, Chironomus riparius, Tubifex spp., and Lumbriculus variegatus. Eh profiles show diurnal rhythms that correspond to D) solar radiation.
Detritus processing, measured as DECOTAB mass loss, was substantially higher in the treatments containing invertebrates as compared to the control treatment without invertebrates (Fig 2D), in which DECOTAB mass loss was significantly higher in the treatment containing [Tubifex spp. and A. aquaticus], as compared to the invertebrate combination [Tubifex spp., A. aquaticus, C. riparius, L. variegatus and G. pulex] (One-Way ANOVA, Tukey-HSD $p = 0.008$). The residuals (Observed - Expected) were significantly higher than 0 in the invertebrate treatment [Tubifex spp. and A. aquaticus], but were almost zero in the other invertebrate treatments (Fig. 4B), suggesting a significant enhanced DECOTAB mass loss in the [Tubifex spp. and A. aquaticus] combination as compared the single species incubations, while no interaction effects were observed in the other invertebrate treatments.

\[
\text{Fig. 4. Residuals (observed – expected calculated from single species incubations) for Biogenic Mixing Depth (A) and DECOTAB mass loss (B), testing the null hypothesis that no interaction between invertebrate species occurred, i.e. residual equaled 0 \text{ (* indicates that residuals significantly deviates from 0, one sample T-test, } n = 5, p < 0.05).}
\]
Discussion

Previous studies that examined the effects of invertebrate activity on microbial (functional) diversity in laboratory microcosms (Mermillod Blondin and Rosenberg 2006; Navel et al. 2010; Bertics and Ziebis 2009) clearly revealed that sediment reworking by invertebrates shapes the composition of the benthic microbial community. However, in the present study, the functional bacterial composition (as represented by the number of substrates used) and the overall activity were variable and different treatments were not statistically separated. This suggests that the effects of sediment reworking on the functional composition of the microbial community were overruled by factors unrelated to invertebrate bioturbation. Several studies have demonstrated that solar radiation can have profound effects on both the structural and functional composition of the bacterial community due to e.g. detrimental effects on DNA (Denward et al., 1999; Piccini et al., 2009). It is likely that solar radiation overruled invertebrate-bacterial interactions in our mesocosms. Effects of solar radiation showed up to strongly impact diurnal changes of the sediment redox conditions in all mesocosms. Although the mechanisms underlying this observation remain uncertain, diurnal rhythms are typically visible in continuous redox potential measurements (e.g. Vorenhout et al., 2004, 2011). It has been demonstrated that redox condition is an important driver for bacterial community structure and activity (Bespalov et al., 1996; Bertics and Ziebis, 2009; Hunting and Van der Geest, 2011; Hunting and Kampfraath, 2013). Since sediment redox potential was similarly affected by solar radiation in all mesocosms, it is possible that a combined effect of solar radiation and subsequent changes in sediment redox potential was converging bacterial community structure in this study. Although this remains speculative and requires further examination, this outcome suggests that the frequently observed influences of invertebrates on bacterial diversity and activity in laboratory studies (Mermillod-Blondin, 2002; Nogaro et al., 2009; Hunting et al., 2012) can become less prominent in the presence of solar radiation, especially in shallow waters.

Sediment reworking by invertebrates resulted in elevated redox potentials within the upper layers of the sediment, reflecting an increased biogenic mixing depth (BMD) (Solan et al. 2004; Birchenough et al. 2012). Increasing diversity from 2 species to 3 species increased BMD, suggesting that increasing community complexity results in altered and wider distributions of the infaunal community and inherent biogeochemical characteristics of the sediment. The observed increase in biogenic mixing is very likely the result of infaunal organisms burrowing deeper in the sediment in response to epifaunal presence. However, increasing the number of species to 5, while maintaining the same number of bioturbation types, did not alter the BMD, suggesting that geochemical
characteristics of the sediment rely on functional diversity rather than species richness. Differences in invertebrate community structure are often visible in the geochemical characteristics of soft bottom sediments (e.g. Solan et al., 2004; Mermillod-Blondin and Rosenberg, 2006; Hunting et al., 2012). However, significant interaction effects were observed in our multispecies invertebrate mesocosms as compared to the single species incubations. BMD in multispecies assemblages tend to be higher than predicted from BMD’s derived from single species incubations, suggesting that invertebrate species interactions have strong positive, yet unpredictable effects on the redox conditions of the sediment.

Cellulose decomposition, in the present study measured as DECOTAB mass loss, is increasingly used as a sentinel substitute of plant litter decomposition and to evaluate anthropogenic impacts (pollution, habitat modification, etc.) on ecosystem functioning in natural environments (Gessner and Chauvet 2002; Tieg et al. 2007; Young and Collier 2009; Imberger et al. 2010). The majority of studies concentrated on the use of commercially available cotton strips (e.g. Boulton and Quinn 2000 Young et al. 2008; Jenkins et al., 2013). Instead, we used the newly developed DECOTABs composed of 75% cellulose (Kampfraath et al. 2012). However, although it was demonstrated that DECOTABs were readily consumed by the shredding isopod A. aquaticus and by the collector-gatherer C. riparius, the extent to which they could be used to develop functional metrics for assessing biodiversity losses remained uncertain (Kampfraath et al., 2013). In this study, DECOTAB mass loss in the treatment containing 2 species revealed some overyielding, pointing to complementary mechanisms at very low levels of diversity. However, DECOTAB mass loss in multispecies assemblages containing 3 and 5 species could be predicted based on bioturbator-specific responses in invertebrate single species laboratory experiments, in which DECOTAB mass loss appeared to rely mainly on shredder (A. aquaticus and G. pulex) abundances. This suggests that biodiversity effects on ecosystem functioning were again relying on the functional composition rather than species richness. Although diversity effects of invertebrates and their activities on decomposition are often considered difficult to predict (e.g. Jonsson and Malmqvist 2000; McKie et al. 2008, 2009), DECOTAB decomposition was directly related to functional diversity in our mesocosms, especially at higher levels of diversity. This suggests that DECOTABs provide a promising tool to assess decomposition in relation to the (functional) composition of invertebrate communities.

Our study simultaneously evaluated functional metrics that reflect distinct components of the detrital food web in response to invertebrate community structure. Our results contribute to the notion that different ecosystem processes respond differently to changes in biodiversity (e.g. Woodward 2009; Reiss et al. 2009), in which the integrated process
(DECOTAB mass loss) seems more predictable than its separate components (bacterial activity and biogenic mixing). Testing functional parameters under natural conditions revealed that abiotic influences (e.g. solar radiation) can mask or even decouple invertebrate-bacterial interactions in the detrital foodweb.

\[1\] Supplementary data related to this chapter will be available online
PART II

Sponge-environment interactions in mangrove stands
Chapter 7

Diversity and spatial heterogeneity of mangrove-associated sponges of Curaçao and Aruba

Published as:
Abstract: Sponges are major epibionts of mangrove roots in the Caribbean. Mangrove sponge communities in the Caribbean mainly consist of species that are typical to this habitat and community compositions often differ from those found on coral reefs nearby. Heterogeneity in species distributions between locations and within locations between roots is often reported. This study quantifies the diversity and abundance of mangrove associated sponges in the inner bays of Curacao and Aruba and correlates variability of regional sponge diversity with environmental variables measured along the surveyed sites. Tannin concentrations vary between mangrove roots, and were correlated to sponge cover as a possible cause for habitat heterogeneity on a smaller scale. A total of 22 species was observed. Heterogeneity in species richness and abundance was apparent, and several sponge species were restricted in their depth of occurrence. Statistical data reduction suggests that sponge diversity may be partly explained by the distance towards adjacent reefs and to the degree of eutrophication, in which the latter is comprised of rate of planktonic respiration, total carbon and turbidity. Tannin concentrations did not determine within locality species heterogeneity as a priori postulated, but were positively related to sponge cover for reasons not yet elucidated.

Key words: epibionts, heterogeneity, mangroves, spatial variations, sponges, tannins
Mangroves form the dominant vegetation in tidal, saline wetlands along (sub-)tropical coasts (Chapman, 1976; Tomlinson, 1986). Mangrove forests are among the most productive ecosystems on earth (Lugo and Snedaker, 1974) and provide nursing grounds for fish (Nagelkerken et al., 2002; Mumby et al., 2004). The fauna associated with mangrove roots is diverse, including crustaceans, bivalves, fishes, ascidians, hydrozoans, bryozoans and sponges (Sutherland, 1980; Fransen, 1986). Mangrove sponge communities in the Caribbean mainly consist of species that are typical to this habitat and in most cases differ from coral reef sponge communities nearby. Despite the characteristic fauna, heterogeneity in species distributions between locations and within locations between individual mangrove roots is often reported (Sutherland, 1980; Bingham, 1992; Farnsworth and Ellison, 1996; Rutzler et al., 2000; Diaz et al., 2004; Wulff, 2004).

Several studies have addressed the spatial variability in species richness and densities in sponge community composition between localities in the Caribbean region. These studies revealed that the distribution of species in the Caribbean may be governed by a combination of physical and biological factors, including larval availability (Bingham, 1992), proximity towards adjacent reefs (Ellison and Farnsworth, 1992; Rutzler et al., 2000), turbidity (Ellison and Farnsworth, 1992; Farnsworth and Ellison, 1996), presence of predators (Pawlik, 1998; Wulff, 2000; Wulff, 2005), exposure to air (Rutzler, 1995), competition among sponges (Engel and Pawlik, 2005; Wulff, 2005) and sub-optimal levels of abiotic variables (Wulff, 2004; Pawlik et al., 2007). However, controversy exists regarding the degree to which biotic and abiotic factors may dominate and at what scales they effectively affect species distributions (Wulff, 2004, 2005; Pawlik et al., 2007). Moreover, most studies that concern the complexity of sponge distributions in the Caribbean region were conducted at offshore cays in Belize, and few studies were carried out in Florida and Venezuela. Consistencies in correlations with environmental variables should be verified and supplemented with quantitative data of mangrove associated sponges at other Caribbean sites in order to gain a more reliable representation of sponge distribution patterns throughout the Caribbean.

Factors that influence sponge distributions on a smaller scale have barely been studied. Sponge distributions within sites can be patchy, in which neighboring roots can harbor different sets of species or may lack any epibiont while neighboring roots are fully covered. Large differences in the composition of the sponge community between roots have been attributed to low recruitment rates, limited larval supply and variable flow rates (Sutherland, 1980; Farnsworth and Ellison, 1996). The importance of competitive interactions among species that occupy the same root has
been demonstrated in Florida. Some mangrove sponges secrete bio-active compounds that mediate overgrowth interactions by inhibition of sponge growth in some species and promotion of overgrowth in other species (Engel and Pawlik, 2000). These aspects contribute to the variability in sponge distributions among roots, but our current knowledge of the underlying mechanisms steering sponge distributions on mangrove roots remains fragmented. Variability between roots is not likely caused by environmental gradients, since at this scale differences are too small to account for differences in the epibiont compositions of neighboring roots. In contrast, roots of the red mangrove, Rhizophora mangle L can have high and variable tannin concentrations (Tomlinson, 1986; Basak et al., 1996). Tannins are a group of secondary metabolites that are known for their anti-microbial and anti-herbivore activity (Cameron and LaPoint, 1978; Alongi, 1987; Scalbert, 1991; Arnold and Targett, 2002, Erickson et al., 2004), and it seems they adversely affect associated macrofaunal abundance (Lee, 1999). Ellison and Farnsworth (1996) reported the presence of mangrove-derived carbon in epibiotic sponges, suggesting that tannins may enter sponge-tissue and influence its physiology or larval settlement, and subsequently alter species distributions.

This study sets out to quantify the diversity of mangrove associated sponges in the inner bays of Curacao and Aruba, and explores correlations between local environmental factors and sponge distributions. The inner bays of Curacao and Aruba show minimal tidal fluctuations and are diverse in shape, size, accessibility and degree of wastewater disposal recruitment (Ebbing, 1997; Siung-Chang, 1997 and references therein), and are expected to differ in degree of nutrient pollution, turbidity and larval recruitment. Tannin concentrations of selected mangrove roots were compared to sponge cover and considered as a possible cause for within locality heterogeneity.

**Methods**

**Study site**

Several studies suggest that sponges are major epibionts of mangrove roots at the outer seaside fringing mangroves of Curacao inner bays (Wagenaar Hummelinck, 1977; van Soest, 1978, 1980, 1984; Fransen, 1986), yet these data are non-quantitative. The mangrove associated sponges of Aruba have not yet been investigated. Several localities within bays of Curacao and Aruba with fringing mangrove forests that were monopolized by the red mangrove *R. mangle* were investigated on the presence of sponges in April and May 2006 (Figure 1). There is a greater magnitude of industrial waste loads (oil, grease, nitrogen, phosphorus suspended solids and biodegradable material) in Curacao compared to
Aruba (AUAI and AUAll: Vistalmar jetty and opposite) (Siung-Chang, 1997 and references therein). Within Curacao there are differences between bays, i.e., St. Jorisbaai (SJI - SJIIV) and Santa Cruzbaai (SC) show no signs of pollution, Fuikbaai (FB) and Spaanse Water (SWI - SWIV) are moderately eutrophicated, and Piscaderabaai (PBI and PBIll) is highly eutrophicated (Ebbing, 1997).
Species diversity and richness

The methodology for determining sponge diversity and richness was modified after Ellison and Farnsworth (1992). Within the chosen localities, approximately 30–40 roots were haphazardly selected along a 50 meter transect. Roots were selected that submerged to a depth of at least 30 cm. Of these lengthier roots, every fifth root was selected for full characterization, i.e., length and diameter of the root, identification of sponge species, number of colonies and sponge coverage as percentage of total examined substrate. Sponge species that had not previously been encountered within a locality during the inventory were sub-sampled and stored in 70% ethanol for identification based on microscopic examination of skeleton structure and spicule morphology and following the nomenclature of Hooper and Van Soest (2002) and Rutzler et al. (2007).

Vertical zonation

Depth of occurrence of sponge individuals was recorded in order to investigate the zonal distribution of sponge species. Zonation patterns were recorded as frequency of occurrence of all species present at depth intervals of 5 cm. Competition-related factors (i.e., overgrowth and allelopathy) may influence the local distribution of species and hence may interfere with other variables (e.g., light availability, grazing pressure) that might otherwise determine spatial patterns. In an attempt to exclude these competition-related factors, an additional assessment was performed in which sponge-species were recorded that either monopolized a root with considerable cover or dominated with at least 60% of the total sponge cover.

Environmental variables

Salinity was determined using a conductivity meter (Millwaukee, SM302), expressed in mS. Conductivity was converted to salinity following the Practical Salinity Scale of 1978 (PSS-78) (Lewis, 1980). Oxygen was measured with an oxygen electrode (One-Cue-systems), read in percentage air saturation on a calibrated digital multimeter and pH was determined using a commercially available test (SeaChem). Turbidity was calculated measuring light intensities (I) at depth (z) and surface (0) using a photometric sensor read in mV by a digital multimeter. Obtained values were converted to the vertical extinction coefficient (η) following Lambert-Beer’s equation: \(I_z = I_0 e^{-\eta z}\). Nitrite (NO₂⁻), nitrate (NO₃⁻) and the total ammonia content (NHtot) were determined using a commercially available test (SeaChem), and combined, representing dissolved inorganic nitrogen (DIN). Silicate (Si) was determined using a similar test. The concentrations of total organic carbon (TOC) and inorganic carbon (TIC) present in the water were quantified using a TOC-analyzer (model 700, O・I・Analytical).
This type of analyzer acidifies TIC and oxidizes TOC to form carbon dioxide, which, in turn, is detected in a non-dispersive infrared analyzer (NDIR). Samples were stored as soon as possible at 4°C until analysis. Reaction time was extended to 35 minutes. The rate of planktonic respiration was determined using an oxygen electrode (OneCue-systems), read in percentage air saturation on a calibrated digital multimeter. Water was sampled in plastic 1 L bottles wrapped in aluminum foil to prevent oxygen production. Bottles were kept in the water to maintain in situ temperature. Decrease of oxygen concentrations was followed for one hour, in which a period of steady abating was used to calculate the rate of respiration.

**Tannin analysis**

The tannin content was determined for roots of *R. mangle* that were either fully overgrown with sponges or had no sponge overgrowth in order to test whether tannins relate to sponge presence. Five roots were sampled for each group in three different bays: Spaanse Water (SWII), Piscadera Baai (PBI) and Aruba (AUAI), and stored immediately at -20 °C until analysis. All materials used for this analysis were wrapped in aluminum foil to prevent photo-oxidation. All chemicals used for this analysis were analytical grade or higher. Tannin contents of the root samples of *R. mangle* were extracted from both the outer tissue, the periderm, and the remaining inner tissue. The periderm was ground in liquid nitrogen; the inner tissue was pulverized in a grinder (Janke & Kunkel, IKAWERK) and subsequently ground in liquid nitrogen. Tannins were extracted as described by Hagerman (1988) using 0.5 mL 70% aqueous acetone on 100 mg sample and an extraction time of 100 minutes. Samples were subsequently assayed as described by Hagerman (1987). Extracts were put in 8 μL aliquots on Petri-dishes containing 10 g.L⁻¹ agar and 1 g.L⁻¹ Bovine Serum Albumin (BSA) fraction V (Merck, > 97%), dissolved in buffer consisting of 0.05 M glacial acetic acid, 60 μM ascorbic acid, adjusted to pH 5 with 2 M NaOH. Plates were incubated at 30 °C for 96 hours. Extracts diffusively migrated within the gel and tannins precipitated upon contact with BSA. The diameter of the resulting ring was measured, squared and expressed as albumin complexing capacity (ACC).
Table 1. Total abundance of sponges as percentage cover of the total examined substrate and corresponding biodiversity measures. Abbreviations of study sites (PB I-AUA II) as presented in Figure 1.

<table>
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<th>Species</th>
<th>Sponge-cover (%)</th>
<th>PB I</th>
<th>PB II</th>
<th>SW I</th>
<th>SW II</th>
<th>SW III</th>
<th>FB</th>
<th>AUA I</th>
<th>AUA II</th>
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<td>Halichondria (Halichondria) magniconulosa</td>
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<td>Halichondria (Halichondria) melanodocia</td>
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<td></td>
<td>0.15</td>
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<tr>
<td>Haliclona (Soestella) caerulea</td>
<td></td>
<td>0.19</td>
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<td>Haliclona (Rhizoniera) curacaoensis</td>
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<td></td>
<td>0.04</td>
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<tr>
<td>Haliclona sp. indet.</td>
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<td></td>
<td></td>
<td>0.13</td>
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<tr>
<td>Iotrochota birotulata</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>1.76</td>
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<td>Ircinia strobilina</td>
<td></td>
<td></td>
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<td></td>
<td>0.73</td>
<td></td>
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<td>Mycale (Zygomycate) angulosa</td>
<td></td>
<td>0.03</td>
<td>3.96</td>
<td>0.09</td>
<td></td>
<td></td>
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<td>Mycale (Aegopoglia) carmigropila</td>
<td></td>
<td>1.6</td>
<td>0.41</td>
<td>2.01</td>
<td>3.17</td>
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<tr>
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<td>5.09</td>
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<td>5.04</td>
<td>4.85</td>
<td>3.98</td>
<td>1.67</td>
<td>5.34</td>
<td>4.73</td>
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<tr>
<td>Tedania (Tedania) ignis</td>
<td></td>
<td></td>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>0.36</td>
</tr>
<tr>
<td>Shannon index</td>
<td></td>
<td>1.523</td>
<td>1.904</td>
<td>1.31</td>
<td>1.111</td>
<td>1.432</td>
<td>2.032</td>
<td>1.46</td>
<td>0.0</td>
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<tr>
<td>Total number of colonies</td>
<td></td>
<td>63</td>
<td>18</td>
<td>57</td>
<td>38</td>
<td>42</td>
<td>59</td>
<td>43</td>
<td>18</td>
</tr>
<tr>
<td>Total number of species</td>
<td></td>
<td>8</td>
<td>3</td>
<td>10</td>
<td>4</td>
<td>7</td>
<td>11</td>
<td>8</td>
<td>1</td>
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<tr>
<td>Total sponge cover (%)</td>
<td></td>
<td>20.96</td>
<td>22.24</td>
<td>18.81</td>
<td>12.81</td>
<td>8.88</td>
<td>17.58</td>
<td>8.73</td>
<td>0.36</td>
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<tr>
<td>Rarified species richness (n = 18)</td>
<td></td>
<td>5.63</td>
<td>2.96</td>
<td>7.42</td>
<td>3.94</td>
<td>5.60</td>
<td>7.85</td>
<td>6.45</td>
<td>-</td>
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<tr>
<td>95 % CL High</td>
<td></td>
<td>7</td>
<td>3</td>
<td>9</td>
<td>4</td>
<td>7</td>
<td>10</td>
<td>8</td>
<td>-</td>
</tr>
<tr>
<td>95 % CL Low</td>
<td></td>
<td>4</td>
<td>2</td>
<td>5</td>
<td>3</td>
<td>4</td>
<td>6</td>
<td>4</td>
<td>-</td>
</tr>
<tr>
<td>Evenness (PIE)</td>
<td></td>
<td>0.93</td>
<td>0.59</td>
<td>0.85</td>
<td>0.79</td>
<td>0.30</td>
<td>0.68</td>
<td>0.98</td>
<td>-</td>
</tr>
<tr>
<td>Number of roots investigated</td>
<td></td>
<td>29</td>
<td>38</td>
<td>40</td>
<td>28</td>
<td>33</td>
<td>34</td>
<td>35</td>
<td>27</td>
</tr>
<tr>
<td>Investigated substrate area (cm²)</td>
<td></td>
<td>9903</td>
<td>13516</td>
<td>14372</td>
<td>8870</td>
<td>9065</td>
<td>14969</td>
<td>1225</td>
<td>9351</td>
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</tbody>
</table>

Statistical analysis

Similarity between localities was analyzed using a multivariate cluster technique, comprised of Euclidean distance and Ward’s minimum variance amalgamation method (STATISTICA v.7.0.). Sponge diversity was expressed as the Shannon index $H'$, as this index subsumes species richness and abundance into a single value. $H'$ was subsequently related to environmental variables by performing linear regression on single variables and co-correlating variables grouped by principal component analysis (PCA) (SPSSR v.10.0.). Data obtained in this study were simulated for sample-based rarefaction on algorithms provided by Ugland et al. (2003), which reveals differences in species density. Ecosim v.7.72. (Gotelli and Entsminger, 2006) was used to simulate individual-based rarefaction curves and the corresponding approximated 95% confidence intervals which allows for comparison between species-richness. Evenness (PIE) (Hurlbert, 1971) was also determined using Ecosim v.7.72. following the rarefaction principle, i.e., a random sample of individuals is drawn from a
given species distribution to estimate sampling effects for the index and provides the probability that two randomly selected individuals will belong to different species. Zonation patterns were assessed performing a one-way ANOVA. Differences in tannin content in relation to sponge coverage were detected performing single $t$-tests. Inferential statistics were computed in Matlab v.7.0.

Fig. 2. Dendrogram of investigated localities based on Euclidian distance and Ward’s method. Locations within bays are encoded as presented in Figure 1. Analysis presents 3 separate clusters in which sites located near each other do not cluster, thereby revealing local heterogeneity.
Fig. 3. Vertical distribution of six sponge species associated with mangrove roots focusing on sponge species dominating or monopolizing roots. Means are given with corresponding standard deviation. Regarding the upper limits of occurrence, two species (G. papyracea and M. carmigropila) are bound to deeper areas compared to the other species (p < 0.05, n = 6). Illustration of mangrove community is taken from Fransen (1986).

Abbreviations: Ca, Clathria sp. indet; Mm, Mycale (Carmia) microsima-tosa; Tm, Tedania (Tedania) ignis; De, Dysidea etheria; Gp, Geodia papyracea and Mc, Mycale (Aegogropila) carmigropila.
Results

**Sponge diversity and richness**

The inner bays of Curacao and Aruba yielded a total of 22 sponge species at eight localities, which differed in number of colonies, species richness, percentage cover and composition of the community (Table 1). Nineteen different species were found at Curacao compared to eight at Aruba. Localities showing the higher diversity were Fuikbaai (11 species) and Spaanse Water (SWI - ten species; all localities of Spaanse Water combined yielded 13 species). Some species were regularly encountered (e.g., *Tedania (Tedania) ignis* (Duchassaing and Michelotti), *Mycale (Carmia) microsigmatosa* Arndt and *Dysidea etheria* De Laubenfels), whereas some species (e.g., *Chelonaplysilla erecta* Carter) were found only once during this inventory. No sponges were present in Jan Thiel Baai, St. Jorisbaai, Santa Cruzbaai and SWIV. Cluster analysis revealed variability in faunal composition among localities, whereas neighboring sites within the same bay did not cluster (Figure 2). Except for Fuikbaai, all sample sizes (28-40 roots) seemed adequate, as rarefaction curves revealed their asymptote (data not presented). Rarifying the data revealed differences in tannin contents of roots of *R. mangle* collected in Curacao (PBI and SWII) and Aruba (AUAI) expressed as Al-bumin Complexing Capacity (ACC). Roots covered with sponges have consistently higher tannin concentrations, as compared with roots without sponge cover (n = 5 in all groups, asterisks indicate significant t-test statistics; PBI: p < 0.01; SWII: p < 0.05; AUAI: p < 0.05). There are no significant differences (p = 0.95) in tannin concentrations between the outer tissue, the periderm (Pd), and the remaining inner tissue (Ed).

![Tannin contents of roots of *R. mangle* collected in Curacao (PBI and SWII) and Aruba (AUAI) expressed as Al-bumin Complexing Capacity (ACC). Roots covered with sponges have consistently higher tannin concentrations, as compared with roots without sponge cover (n = 5 in all groups, asterisks indicate significant t-test statistics; PBI: p < 0.01; SWII: p < 0.05; AUAI: p < 0.05). There are no significant differences (p = 0.95) in tannin concentrations between the outer tissue, the periderm (Pd), and the remaining inner tissue (Ed).](image-url)
species density between most of the investigated sites and showed significant ($p < 0.05$) differences in species richness between several localities, including sites located within a single bay (Table 1).

### Vertical zonation

Sponge species were more or less equally distributed over the examined root substrate if all sponge distributions were taken into account (data not presented). However, when competition related factors are excluded from the analysis (i.e., considering only roots monopolized or dominated by a single sponge species), differences become apparent for some species (Figure 3). These differences were significant ($p < 0.05$, $n = 5$) with respect to the upper limit of occurrence, in which *Geodia papyracea* Hechtel and *Mycale (Aegogropila) carmigropila* Hajdu and Rutzler seemed restricted to deeper regions of the root. No differences were found with respect to the lower limits of their occurrence ($p = 0.35$, $n = 5$).

### Table 2. Physico-chemical variables of the different localities

<table>
<thead>
<tr>
<th>Variable</th>
<th>Locality:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>PB I</td>
</tr>
<tr>
<td>pH</td>
<td>8.05</td>
</tr>
<tr>
<td>Oxygen</td>
<td>85</td>
</tr>
<tr>
<td>DIN</td>
<td>0.12</td>
</tr>
<tr>
<td>TOC</td>
<td>1.804</td>
</tr>
<tr>
<td>TIC</td>
<td>27.23</td>
</tr>
<tr>
<td>RR</td>
<td>38</td>
</tr>
<tr>
<td>Si</td>
<td>0.5</td>
</tr>
<tr>
<td>Turbidity</td>
<td>1.65</td>
</tr>
<tr>
<td>Salinity</td>
<td>35.1</td>
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<tr>
<td>Distance</td>
<td>1150</td>
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</tbody>
</table>

### Table 3. Statistics of single environmental variables and co-correlating variables grouped by PCA factor analysis for apparent correlations with sponge diversity.

<table>
<thead>
<tr>
<th>Variables</th>
<th>Independent variable</th>
<th>Dependent variable</th>
<th>Statistics</th>
<th>$r^2$</th>
<th>p-value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Distance to reef</td>
<td>Shannon index</td>
<td>$-0.0321 \times +2.0531$</td>
<td>0.5326</td>
<td>$&lt; 0.05$</td>
<td></td>
</tr>
<tr>
<td>(Factor analysis)</td>
<td>Total carbon</td>
<td>$-147.88 \times +272.93$</td>
<td>0.7537</td>
<td>$&lt; 0.05$</td>
<td></td>
</tr>
<tr>
<td>Turbidity</td>
<td>Respiration rate</td>
<td>$-314.81 \times +562.96$</td>
<td>0.5176</td>
<td>0.068</td>
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</tr>
<tr>
<td>Respiration rate</td>
<td>Shannon index</td>
<td>$-0.0652 \times +4.0127$</td>
<td>0.6918</td>
<td>$&lt; 0.05$</td>
<td></td>
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<tr>
<td>Respiration rate</td>
<td>Total Carbon</td>
<td>$1.3558 \times +2.5036$</td>
<td>0.2787</td>
<td>0.21</td>
<td></td>
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<tr>
<td>Factor</td>
<td></td>
<td>$-0.291 \times +1.9026$</td>
<td>0.4262</td>
<td>0.113</td>
<td></td>
</tr>
</tbody>
</table>
Environmental variables

Environmental variables are listed in Table 2. The localities differed greatly in their distance to the reef, dissolved oxygen levels and rate of planktonic respiration, and there were minor differences in turbidity, dissolved inorganic nitrogen and carbon. Sites were very similar in salinity and pH. Statistical data reduction by factor analysis suggests that species richness may be partly explained by distance to nearest reef and by eutrophication, in which the latter represents a linear combination of respiration rate (RR), turbidity and total carbon (TC) (statistics and equations provided in Table 3). Distance to adjacent reefs did not correlate with single or combined eutrophication components \( (p > 0.8) \). Turbidity, RR and TC partly cocorrelated as revealed by principal component analysis, in which there was a clear relation between RR and sponge diversity, RR and turbidity, and turbidity and TC. There was no apparent relation between RR and TC, which caused the PCA-derived factor analysis to result in a single, insignificant factor.

Tannin analysis

Tannin contents of mangrove roots were significantly \( (p < 0.05) \) higher in roots that were covered with sponges compared to roots that were not covered with sponges (Figure 4). The tannin content was considerably higher in roots collected in the Piscadera Baai compared to roots sampled in the other bays (Figure 4). No differences \( (p = 0.95) \) were found between the tannin contents of the outer root tissue, the periderm (Pd), and the remaining inner tissue (Ed) (Figure 4).

Discussion

The majority of sponge species found in this study are typical mangrove sponges known to inhabit mangrove roots, seagrass beds and adjacent shallow reefs, whereas a few species are usually found in a reef environment. Most species found in this inventory were previously reported on mangrove roots in the Caribbean (e.g., Sutherland, 1980; Wulff, 2004). Species were patchily distributed within transects and several species that dominated at one site were completely absent at another site. This phenomenon is consistent with earlier reports on sponge community structures in mangroves (Rutzler et al., 2000; Diaz et al., 2004; Wulff, 2004).

Results on environmental variables in this study reflect single point measurements and fail to reveal the temporal variation of these parameters, which may be large seasonally and even daily. Interpretations of apparent relations should therefore be restricted to environmental variables that show discernible gradients. Statistical data reduction
suggests that the diversity patterns found in this study may relate to two variables: distance to the nearest reef and eutrophication.

The correlation between sponge diversity and proximity towards adjacent reefs validates earlier field observations in Belize, including an increased species richness with decreased distance to well developed coral reefs in off shore cays (Rutzler et al., 2000), and increased species richness in a coast to barrier reef transect (Ellison and Farnsworth, 1992). A similarity between this study and the Belizean survey of Rutzler et al. (2000) is that localities close to reefs harbored more species typical of nearby reefs (e.g. Desmapsamma anchorata Carter, Ircinia strobilina Lamarck and lotrochota birotulata Higgin), while localities further from the reef are largely comprised of species typical to mangrove habitats (e.g. Tedania (Tedania) ignis, Lissodendoryx (Lissodendoryx) isodictyalis Carter, Haliclona (Soestella) caerulea Hechtel, Halichondria (Halichondria) magniconulosa Hechtel). Sponge larvae are incapable of traveling large distances and it has been demonstrated in Florida and Belize that epifaunal communities are partly structured by proximities to source populations and larval life span (Bingham, 1992; Farnsworth and Ellison, 1996). This may suggest that the reef partly functions as a larval pool, rather than that the mangrove habitat is self-sustaining. Sponge community composition varies or clusters with respect to proximity towards the reef (Figure 2) yet the clustering is not fully consistent. In some cases, localities close to the reef are more strongly related to localities that lie furthest from the reef, which points to mangrove-derived recruitment as well. This mechanism would parallel other areas (e.g., the Mediterranean); in which larval recruitment is also partly responsible for observed patterns in sponge community structures (Uriz et al., 1998).

Although free-living phases of sponge larvae are short and dispersal abilities are limited, communities should be homogenously distributed after multiple generations. Distributions patterns are therefore controlled by a combination of factors. It has been shown by others that turbidity plays a pivotal role in structuring epifaunal communities (Bingham, 1992; Ellison and Farnsworth, 1992). In this study, statistical data reduction of combined parameters suggests a pattern of decreasing diversity with increasing eutrophication. Previous work has demonstrated the adverse effects of eutrophication on sponges under laboratory conditions (Roberts et. al., 2006) and in other habitats, including reefs and kelps (Hindell and Quinn 2000; de Voogd et al., 2006).

The importance of abiotic factors affecting sponge distributions in coastal and estuarine mangrove habitats has been emphasized in earlier studies, in which decreased species richness was attributed to large variation in temperature, salinity and tidal range (Ellison and Farnsworth, 1992; Rutzler, 1995). In addition, transplantation of reef sponges to
mangrove habitats was successful in off-shore cays with similar abiotic conditions in Belize (Wulff, 2005), but resulted in the death of transplants when reef sponges were transplanted to coastal mangrove habitats and off-shore mangroves in Belize and Florida (Ellison and Farnsworth, 1992; Wulff, 2004; Pawlik et al. (2007). The inability of reef sponges to survive transplantation to coastal habitats has been attributed to sub-optimal levels of abiotic variables in coastal and estuarine mangrove habitats (Pawlik et al., 2007).

The tannin concentrations of mangrove roots did not meet our expectation, as roots have significantly higher tannin contents when they are covered with sponges as compared with roots without sponge cover. In general, tannins are considered to function as anti-grazing substances, which may suggest that mangrove roots do not favour sponge coverage. However, another study provided evidence that this association is beneficial for roots, as epibiotic cover protects roots from isopod invasion, coinciding with increased growth rates of roots (Ellison and Farnsworth, 1990). In this study, roots were not examined on the extent of isopod presence, yet all sampled roots appeared rather fragile and soft to the touch, indicating that roots were damaged by isopods (Ellison and Farnsworth, 1990). Ellison et al. (1996) posed the presence of a facultative mutualistic relationship between the red mangrove and the sponge species Haliclona (Reniera) implexiformis Hechtel and Tedania (Tedania) ignis. They found a nutrient exchange between both organisms through fine rootlets, in which the sponge obtains organic carbon from the roots, and mangrove roots take up excretory nitrogen from the sponge. However, formation of such rootlets is restricted to only a few species (Ellison et al., 1996) and the release rates of excretory nitrogen are highly variable between species and depend on the size of non-photosynthetic symbiotic bacterial populations and the nature of photosynthetic symbionts (Corredor et al., 1988).

Although it is apparent that roots have increased tannin levels when covered with sponges, it remains unknown whether this increase has consequences for sponge physiology or the association, and whether tannins in roots of R. mangle are produced to act against sponge tissue. These aspects are subject of further investigation. There is evidence that the protein-binding potential of tannins is greatly reduced when the pH is greater than 7,5 (Martin and Martin, 1983, Martin et al., 1985), and the pH always exceeded 7,5 at sites harbouring sponges during this investigation. The ineffectiveness of tannins under similar conditions has also been shown by Benner et al. (1986), who provided evidence that tannin leachates of mangroves did not reduce microbial degradation. In addition, the production of tannins as chemical defence by the brown algae Fucus vesiculosus sometimes fails to affect herbivores and it is hypothesized that
other metabolites may be a confounding factor in identifying chemical defences (Kubanek et al., 2004). Alternatively, tannins may be redox active to metal ions and alter metal uptake, availability and toxicity, and the mangrove polyphenolics can have anti-oxidant properties (A.E. Hagerman, pers. comm., 2006). The greater part of the non-precipitating flavonoids in leaves of *R. mangle* is comprised of Quercetin, a compound highly effective in scavenging oxy-radicals (Kandil et al., 2004). Tannin leachates may also provide a carbon source for sponges. An increased tannin content may then imply favourable conditions for sponges and larvae seeking a proper substrate to settle on and may favour roots with higher concentrations of tannins in the surrounding water. Future research efforts should elucidate whether increases in tannin concentrations are induced by newly colonizing sponge species or increased tannin concentrations in the surrounding water may act as a cue for attracting sponge larvae. This may provide more insight in the role of tannins in the distribution of mangrove associated sponges.

This study aimed to quantify diversity of mangrove associated sponges in bays of Curacao and Aruba and to correlate complexity in community structure with environmental variables. Observed patterns validate earlier observations that a combination of physical and biological factors, proximity to source populations and water quality seem important in controlling epifaunal sponge distributions on a larger scale in Caribbean mangrove ecosystems. Heterogeneity in species distributions between roots remains unresolved in many aspects, although data presented here suggest that differences in tannin content may influence the structure of Caribbean sponge communities.
Chapter 8

Mangrove-sponge associations:
a possible role for tannins

Published as:
Abstract: A positive correlation between sponge coverage and tannin concentrations in prop roots of *Rhizophora mangle* L. has previously been reported. However, the ecological role of tannins within the mangrove sponge association remains speculative. This study investigated whether tannins play a role in sponge recruitment and assessed tannin and polyphenol production in *R. mangle* roots in response to sponge colonization. We demonstrated in a field experiment using artificial substrates with different tannin concentrations that tannins are positively involved in larval recruitment of the sponge *Tedania ignis*, and that roots significantly enhanced tannin and polyphenolic content in response to natural and experimental sponge fouling. Differential recruitment in response to tannins may have been the result of a behavioral response in sponge larvae. It is also possible that tannins affected the structure of the fouling microbial biofilm on the artificial substrate, or tannins affected the post-settlement dynamics of sponge recruits. Elevations in concentrations of tannins and polyphenolic compounds upon coverage with sponges, combined with differential recruitment of *T. ignis* in response to differences in tannin concentrations, may indicate a positive feedback in recruitment. This may in part explain the typical heterogeneity in sponge coverage and community composition among roots.

Keywords: Substrate selection, Sponges, Tannins, Polyphenols, Mangroves, Recruitment
Sponge communities in mangrove systems consist of species typical to this habitat but are spatially heterogeneous, i.e. neighboring roots can vary greatly in their sponge coverage and composition. This variability can partly be attributed to low recruitment rates, limited larval availability and interactions among sponges (Sutherland 1980; Bingham, 1992; Engel and Pawlik 1995), or may be the result of chemically induced interactions between sponges and mangroves. Tannins and polyphenolic compounds constitute the greater portion of carbon leachates from mangrove leaves (Maie et al. 2006) and concentrations of tannins and polyphenols may vary depending on tissue, growth stage and environmental conditions (Northup et al., 1998; Lin et al., 2006). A positive correlation between sponge coverage and tannin concentrations in the roots of *R. mangle* was previously reported, in which roots with considerable sponge cover (>40%) contained elevated tannin concentrations compared to unfouled roots (Hunting et al., 2008). This suggests that tannins and polyphenolic compounds in general are potentially involved in the mangrove sponge association.

The mechanism responsible for the observed relation remains speculative since the ecological functioning of tannins is ambiguous. Firstly, it has been speculated that tannins act as a settling cue for sponge larvae (Hunting et al., 2008) or provide a carbon source for epibiontic sponges (Ellison et al., 1996) as leached DOC precipitates are also a source of particulate food for metazoans (Baylor and Sutcliffe, 1963; Kuznetsova et al., 1984). Secondly, increases in tannin concentrations may be a physiological response of mangrove roots to sponge fouling. A substantial number of studies have suggested that grazing and fouling are strongly correlated to increases in polyphenol concentrations in which polyphenols may form recalcitrant complexes that are resistant to biodegradation, thereby reducing palatability and inhibiting growth of fouling organisms (e.g. Schmitt et al. 1998). Thirdly, sponge presence may positively or negatively affect nutrient availability for *R. mangle* roots, thereby causing differences in secondary metabolite production. It has been hypothesized that excess carbon may be allocated to carbon rich secondary metabolites in the absence of nutrients (Bryant 1987), while several studies have provided evidence that nutrient enrichment enhances production of total phenolics and tannins in *R. mangle* (Feller 1995; Feller et al. 2003; Feller & McKee 2003).

The objective of this study was to evaluate whether tannins play a role in sponge recruitment and whether roots of *R. mangle* enhance production of tannins and total phenolics in response to sponge colonization. These aspects were addressed by performing *in situ* recruitment and translocation experiments.
Methods

Study site

This study was conducted within Spaanse Water (12°04’21.78”N, 68°51’38.87”W), an inner bay of Curaçao, N.A., Southern Caribbean sea (Figure 1), in the period of March until June 2008. Detailed information on physico-chemical characteristics is provided elsewhere (Hunting et al. 2008: and references therein). In brief, the site is moderately eutrophic with low turbidity. Distance to the nearest reef is 2.2 km. Salinity is around 35 psu and pH is around 8. Tidal ranges are approximately 10 cm and resident sponge communities do not emerge during low tide. Sponge coverage is on average little over 10 percent of the total root substrate.

Recruitment experiment

The role of tannins in recruitment of sponge larvae was assessed with artificial substrates made from polymeric gels. This method proved useful in studies focusing on the retention of larval settlement (e.g. Henrikson & Pawlik 1995; Browne & Zimmer 2001). Tannins embedded in a matrix of agar diffuse into the overlying water. A total of 30 mimicry gels (surface area 88 cm², volume 440 cm³; 20 g.L⁻¹ agar and 60 μM ascorbic acid) consisted of 3 different treatments containing either 0, 0.3 or 1.8 nM tannic acid (purity > 96%, Sigma-Aldrich). Although the majority of phenolic compounds in woody plants are condensed tannins we used tannic acid as a representative of hydrolyzable tannins, the second major group of phenolics that occur primarily in young, rapidly growing tissues (e.g. prop roots of *R. mangle*) of woody plants (Haukioja et al., 1998). The mimicry gels were vertically installed with plastic rope throughout the mangrove fringes at ± 0.8 m depth in a random fashion in order to minimize the effect of variable physico-chemical conditions (e.g. flow conditions). Gels were placed within cages (mesh-size 1 cm²) to prevent spongivory. The average leaching rates of tannic acid from agar gels were approximated from a 7 week incubation in aquaria containing artificial seawater with starting concentrations of 0.3 and 1.8 nM. Leaching rates decreased about 40% over the course of the experiment, but were still detectable after 7 weeks. The average leaching was 4 (± 0.26 s.d.) mg.C.d⁻¹ and 15 (± 0.54 s.d.) mg.C.d⁻¹, respectively. The average leaching rates are comparable to leaching rates of total phenols (on average 2.4 mg.C.d⁻¹) reported by Maie et al. (2006), and dissolved organic carbon (on average 10 mg.C.d⁻¹) reported by Camilleri and Ribi (1986). Conversion of reported leaching rates followed an empirical relation between root dry mass and root length reported by Ellison and Farnsworth (1996). Mimicry gels were collected after 7 weeks and brought to the lab. Gels remained submerged in seawater during transport to prevent exposure to air. Recovered recruits were prepared for microscopy in canada balm. Species
identification was based on microscopic examination of skeleton structure and spicule morphology following the nomenclature of Hooper & Van Soest (2002).

Transplantation experiment

A transplantation experiment with specimens of *Tedania ignis* was performed in order to determine whether sponge fouling can induce enhanced tannin and polyphenol production. Fifteen roots were selected for each of the following treatments: (1) natural root cover, in which sponge coverage exceeded 40 % (primarily *T. ignis*); (2) bare roots that were not covered by any fouling organism during sampling; (3) bare roots used for transplantation of specimens of *T. ignis* (collected from neighboring roots and adjacent benthic substrata) attached with plastic cable ties as described by Ellison et al. (1996), in which we aimed to obtain a coverage that was comparable to treatment 2 (>40%); and (4) bare roots that were wrapped in plankton net (mesh size 500 μm), to allow natural turbidity to occur while preventing larval recruitment and isopod invasion, thereby ensuring that roots remained unfouled over the course of the experiment. The latter treatment served as an extra control for treatment 2, for which there was no guaranteed absence of fouling organisms during the experiment. Mortality of transplants was < 5%. Any losses within the first 2 weeks of the experiment were replaced. Samples (15 cm root segments) were collected after 8 weeks, wrapped in aluminum foil and immediately stored on ice. All samples were stored at -20°C within 1 hour after collection and remained frozen until analysis.

Fig. 1: Map of Curaçao and Spaanse water (inset) showing location of the study site (shaded area).
Analytical techniques

Root samples (15 cm) were freeze-dried and ground with an electric coffee grinder (particle sizes ranged 200 – 300 μm). Tannins and polyphenolic compounds were extracted from 100 mg of ground sample as described previously (Hunting et al. 2008). Analysis of the protein precipitating fraction of tannins is described in detail elsewhere (Hagerman 1987; Hunting et al. 2008). Total phenolics were determined using Folin-Ciocalteu reagents as described by Ragazzi & Veronese (1973). Prior to this assay, 10 μL extracts were put in open aliquots in a flow cabinet to allow vaporization of aceton. Phenolics were subsequently resuspended in 10 μL deionized water and assayed. Absorbance was measured at 740 nm (Nanodrop, ND1000). Tannic acid was used for calibration and results were expressed as Tannic Acid Equivalents (gTAE.gDW⁻¹). Treatments were compared by performing a one-way ANOVA with a Tukey-Kramer post hoc test for multiple comparisons of means (Matlab v.7.0).

Fig 2. Average number of sponge recruits on mimicry gels with different tannin concentrations. Provided are means (± s.e.). Corresponding letters indicate statistical similarity (α < 0.05; n = 10 per treatment).
Results

Sponge recruitment on mimicry gels containing tannic acid is presented in Fig. 2. An average of 7 recruits was recovered from gels with high tannic acid contents (1.8 nM), which was significantly (p<0.05) higher compared to an average of 3 recovered recruit in mimicry substrates that did not contain tannic acid. Figure 2 suggests that there is enhanced recruitment in the lower ranges of tannic acid content (0.3 nM), however, there was no statistical difference between the low concentration treatment and that of the zero and high concentration (1.8 nM) treatments. Next to sponge recruits, the mimicry gels also contained some Bryozoa, and each treatment contained a patchy, yet unspecified microbial biofilm. All recruits were 2 - 3 mm in size. Over 90% of the recruits recovered from the mimicry gels were identified as *Tedania (Tedania) ignis* (Duchassaing and Michelotti) and the remaining species included *Desmapsamma anchorata* (Carter), *Dysidea janiae* (Duchassaing & Michelotti) and *Ircinia felix* (Duchassaing & Michelotti).

Total phenolic and tannin concentrations in mangrove roots of the different treatments are presented in Figures 3A and 3B, respectively. Following an 8 week incubation period, roots covered with sponges contained approximately 20% more phenolic compounds and 22% more tannins than roots that were not covered by sponges. Roots covered with transplants contained significantly higher (p<0.05) concentrations of total phenols, and tannins compared to roots covered with plankton net. Furthermore roots that were naturally covered with sponges expressed a significant (p<0.05) increase in total phenol content compared to roots covered with plankton net.

Discussion

The present study demonstrates that recruitment of the sponge *Tedania ignis* is enhanced when substrates contain higher tannin concentrations. Sponges have short living lecithotrophic larval stages and hydrology and stochasticity are considered the principal factors explaining large scale spatial patterns, while active habitat selection becomes progressively more important at small (< 1m) spatial scales (Pawlik 1992; Mariani et al., 2006). Since roots of *Rhizophora mangle* generally provide the only stable substrate in mangrove ecosystems, substrate localization is a critical process for the successful reproduction of mangrove associated sponges. In order to actively select an appropriate substrate, sponge larva should have the ability to detect and discriminate substrate specific cues. Enhanced recruitment associated with increased tannin concentrations may be the result of a behavioral response of sponge larvae to tannins. However, although larvae of several sponge species are known to actively
Fig. 3. Concentrations of (A) total phenolic compounds (expressed as Tannic Acid Equivalents, TAE) and (B) protein precipitating tannins (expressed as Albumin Complexing Capacity, ACC) of mangrove roots after 8 weeks of incubation with four different treatments in Spaanse Water, Curaçao. Treatments include natural covered roots, natural unfouled roots, roots covered with transplants and roots wrapped in plankton net. Bars indicate means (± s.e.) and corresponding letters indicate statistical similarity (α < 0.05; n = 15 per treatment).
examine and select an appropriate substrate and chemical stimuli are often considered a cue in selective settlement (Maldonado 2006, Whalan et al. 2008), no unequivocal evidence exists that clearly demonstrates chemotaxis and chemical regulation of settlement in sponge larvae. Alternatively, tannins may have altered the structure of the fouling microbial consortium and larvae of *T. ignis* may have subsequently responded to differences in chemical, textural or structural aspects of the microbial biofilm. In addition, differences in tannic acid contents between treatments may have caused differences in post-settlement dynamics. It is possible that higher tannic acid concentrations may have reduced mortality of sponge recruits due to increased nutrient availability or reduced predation. Irrespective of the mechanism, a direct or indirect influence of tannins on sponge larvae may suggest that secondary metabolites of plants and algae play a pivotal role in substrate selection or post-settlement dynamics and henceforth polyphenolic compounds may be involved in structuring epibiont communities. Future research efforts should elucidate whether larvae of *T. ignis* respond directly to dissolved tannins or polyphenolic compounds or indirectly to possible differences in the fouling microbial assemblages. It is also necessary to evaluate the early stages of sponge community assembly and post-settlement dynamics in relation to tannins and polyphenols in order to gain insight in the ecological relevancy of tannins and polyphenols in sponge community patterns.

Sponge colonization promoted tannin and phenol production in *R. mangle* roots. We can not exclude the possibility that elevations in tannin concentration are physiological responses to stress (e.g. injury). However, we speculate that the elevated concentrations of tannins and polyphenolic compounds upon coverage, combined with differential recruitment of *T. ignis* in response to differences in tannin concentrations, may result in a positive feedback in recruitment, i.e. settlement induces elevation in secondary metabolites, which, in turn enhances the number of recruits. This type of mechanism may help explain among root heterogeneity and would complement a facultative mutualism between *R. mangle* and common root fouling sponges as proposed by Ellison et al. (1996). These authors presented evidence that *R. mangle* roots produce adventitious rootlet generally involved in nutrient uptake that ramify tissue of several sponge species, including *T. ignis*. They also found that roots of *R. mangle* obtain nitrogen from sponges and that sponges obtain carbon from *R. mangle* roots, while both sponge and tree exhibit enhanced growth rates upon association. In addition, enhanced eutrophic state and experimental fertilization has been shown to result in elevations of *R. mangle* phenolics (Feller 1995; Feller et al. 2003; Feller & McKee 2003; Hunting et al. 2008). Elevations of phenolic compounds in this study may therefore indicate that
sponge coverage enhances nutrient availability to *R. mangle* roots.

This study aimed to evaluate the role of tannins within the association of sponges and prop roots of the red mangrove *R. mangle*. The results presented here indicate that tannins are directly or indirectly involved in the recruitment of the sponge *T. ignis* and suggest that the ecological role of tannins within the mangrove-sponge association is more complex than previously anticipated.
Chapter 9

Degradation of mangrove-derived organic matter in mangrove associated sponges

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Abstract: Sponge communities found in Caribbean mangroves are typical to this habitat, in which sponge communities are partly endemic and very distinct from sponge communities on nearby reefs. A trade-off between resistance to competitors and predators appears to influence success of individual sponge species in mangrove habitats. We speculate that differences in the symbiotic microbial communities may partly be responsible for these differences, as partial degradation of recalcitrant compounds by tannin degrading microorganisms may enhance palatability and facilitate DOM assimilation in the presence of high concentrations of tannins, thereby improving their competitive capabilities. We tested tannase activity and ability to degrade mangrove-derived dissolved organic matter (DOM) in a random set of sponge species collected from mangrove roots in Curaçao and adjacent reefs. Our results suggest that sponges commonly associated with mangrove roots contain bacteria that are capable of degrading mangrove-derived DOM, while bacterial communities associated with sponges that are more typical to reef environments appear less proficient in degrading mangrove-derived DOM. Host specificity of bacterial endobionts capable of degrading mangrove-derived DOM and the presence of high concentrations of recalcitrant organic compounds may lead to ecological separation between mangrove and reef sponge communities.

Keywords: Dissolved organic matter, Sponges, Bacterial symbionts, Reef, Mangrove roots.
Sponges are the dominant fouling fauna within the epibiontic communities that live on submerged roots of the red mangrove *Rhizophora mangle* (Linnaeus, 1753) throughout the Caribbean. Sponge communities found in most Caribbean mangroves are typical to this habitat, i.e. some species are endemic and the structures of the sponge communities in mangroves and nearby reefs are very distinct. It was recently demonstrated that spongivorous predators can exclude typical mangrove sponges from reef assemblages, while reef sponges are excluded from mangrove sponge assemblages by competition in the absence of predation, suggesting a trade-off in resistance to competitors and predators (Wulff, 2005).

Decomposing mangrove foliage litter as well as leaching of tannins and polyphenolic compounds from mangrove roots are the primary input of organic matter in mangrove ecosystems (Dittmar, 2004; Maie and Jaffe, 2006; Kristensen et al., 2008) and recent evidence clearly demonstrated that mangrove-derived dissolved organic matter (DOM) is the primary carbon source for sponges living in mangrove habitats (Granek et al., 2009). Most sponges form close associations with a wide variety of microorganisms (Taylor et al., 2007), and bacterial symbionts have been shown to play a pivotal role in organic carbon assimilation (de Goeij et al., 2008a,b). However, mangrove-derived DOM consists mainly of tannins and polyphenolic compounds (Maie and Jaffe, 2006). It is well established that tannins are structurally complex and recalcitrant to biodegradation (Field and Lettinga, 1992) and a significant fraction (~50%) of mangrove-derived DOM is relatively resistant to degradation (Koch et al., 2005; Kristensen et al., 2008). Since only a limited number of bacterial and fungal species are able to degrade complex polyphenols and tannins (Bhat et al., 1998), and the structure of endobiontic communities is at least partially host specific in the majority of sponges (Taylor et al., 2007), the structure of the microbial endobiontic community of sponges may be an important component in the macroecology of tropical sponges in the western Atlantic.

It is hypothesized that the presence of tannin-degrading microorganisms within the endobiontic community of mangrove sponges may be partly responsible for the structural differences in reef and mangrove sponge communities as partial degradation of recalcitrant compounds may enhance palatability and thereby facilitate DOM assimilation in the presence of high concentrations of tannins. In contrast, the absence of tannin degraders in the endobiontic community may limit developmental and competitive capabilities of sponges. To begin to test this assumption, we qualitatively explore the presence of tannin-degrading organisms in a random set of species collected from mangrove roots and a nearby reef by assaying tannase activity and evaluate whether endobionts are able to grow on artificial substrate containing mangrove root extracts.
Methods

Sponge and root material

Material was collected in Curaçao, N.A., southern Caribbean, during fieldtrips in May and September 2009. Twenty mangrove sponges were collected from the inner bays Spaanse Water and Piscaderbaai, and fifteen reef specimens were collected at the shallow reefs in front of the research facility of Carmabi (Caribbean Research and Management of Biodiversity) and prepared for analysis as described below. For detailed maps of the sites see Hunting et al., 2008, in press; De Goeij et al., 2008b. Sponge species were identified based on examination of skeleton structure and spicule morphology, in which we followed the nomenclature of Van Soest et al. (2008). Root segments were haphazardly collected in Spaanse Water and Piscaderabaai, wrapped in aluminum foil to prevent photooxidation and stored at 200°C until sample preparation as described below.

Tannase activity

A comparison was made between tannase activity in sponges originating from either mangrove roots or adjacent reefs. Approximately 1 cm³ sponge tissue was sampled and incubated with seawater containing 0.5 g.L⁻¹ tannic acid for 3 hours at ambient temperature. Seawater was subsequently discarded, and samples were stored at -20°C until analysis. Tannase activity was assayed using methyl gallate, MG, as described by Osawa and Walsh (1993). In brief, a subsample of the sponge tissue (0.1 cm³) was added to 20 mL substrate medium (pH 5) containing NaH₂PO₄ buffer (33mM) and MG (20mM). Samples were incubated at 37°C for 24 hours under anaerobic and dark room conditions. Methyl gallate is colorless and turns greenish-brownish when hydrolyzed. Coloration of the medium is judged as a positive result for tannase activity. Negative controls containing either sponge tissue and buffer, or MG and buffer, were included to validate that color formation was due to sponge derived tannase activity and to correct for extraction of sponge pigments.

Degradation of mangrove-derived DOM.

In order to determine whether sponge endobionts are able to degrade mangrove-derived DOM, we inoculated bacterial extracts from sponges on agar containing R. mangle root extract. Sponge endobionts were extracted from subsamples of sponge tissue (0.1 cm³) in a Precellys® 24 lysis/homogenizer (Bertin Technologies, France) using Ø0.5 mm beads and subsequent centrifugation for 30 s at 11000 g. Tannins and polyphenols were extracted from freeze-dried and ground R. mangle roots (40g DW) with 70% aqueous acetone for 48 hours. Extracts were centrifuged (4000
rpm 15 minutes) and pellets were air-dried in a flow cabinet. Extracts were subsequently dissolved in deionized water (1L final volume) containing agar (2 g.L) and NaCl₂ (35 psu) and autoclaved for sterilization. This mixture was poured into 50 mL sterile centrifuge flasks to obtain a total substrate volume of 10 mL. After solidification, we added 1 mL of endobiontic extract and artificial seawater 35 psu NaCl₂ to a final volume of 50 mL. Samples were incubated for 72 hours at 37°C under anaerobic conditions. Negative controls did not contain endobiontic bacteria. Overall respiration was subsequently determined measuring DIC accumulation in the overlying water (TIC-TOC analyzer, OI-analytical). Developed biofilms were examined microscopically for presence of bacteria and fungal hyphae. Viability and activity was determined by measuring electron transport system activity (ETSA) following the reduction of 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl tetrazolium chloride (INT) to formazan (INTF) sensu Smith and McFeters (1997). Developed biofilms were harvested from the agar surface by scraping and redissolved in 200 μL dH₂O. Cell integrity was subsequently disrupted by 15 min sonication at room temperature (Branson, 1510). An aqueous INT solution (200 μL; 1 mM with 0.05% dimethylsulfoxide, DMSO) was added and the samples were mixed and incubated for 2 hours at 20 °C under dark room conditions. Enzyme activity was stopped by adding 500 μL DMSO and measured spectrophotometrically at 490 nm (Shimadzu, 1601-UV). Controls were treated with formalin (final concentration 5%) to correct for abiotic reduction of INT.

Results and Discussion

A total of 15 sponge species were identified from 35 samples (Table 1). Sponge species collected from the mangrove habitat, including Tedania ignis (Duchassaing and Michelotti, 1864), Mycale microsigmatosa (Arndt, 1927), Chelonaplysilla erecta (Row, 1911), Callyspongia pallida (Hechtel, 1965), Haliclona caerulea (Hechtel, 1965) and Dysidea etheria (de Laubenfels, 1936) are commonly found on roots of R. mangle throughout the Caribbean (Voss, 1976; Van Soest, 1978, 1980, 1984). Two species, i.e. Desmapsamma anchorata (Carter, 1882), Ircinia strobilina (Lamarck, 1814) were collected that are known to occur in both habitats (Voss, 1976; Van Soest, 1978, 1980, 1984; Rützler et al, 2000) and were collected from both habitats during this investigation. Scopalina ruetzleri (Wiedenmayer, 1977) and Halisarca caerulea (Vacelet and Donadey, 1987) were collected from the reef, but are known to occur in both the mangrove and reef environment. The remaining species, Aplysina archeri (Higgin, 1875), Aiolochroia crassa (Hyatt, 1875), Pandaros acaenthifolium (Duchassaing and Michelotti,1864), Cribrochalina vasculum (Lamarck, 1814) and Callyspongia
Table 1: Tannase activity, bacterial activity and rates of mangrove derived organic matter degradation of individual sponge species collected from mangrove roots and adjacent reefs (Provided are mean ± S.D). Previously published data on the presence of microbial symbionts is included.

<table>
<thead>
<tr>
<th>Species</th>
<th>(mg/d)</th>
<th>DIC</th>
<th>ETSA</th>
<th>Tannase activity</th>
<th>Natural habitat</th>
<th>Sampled habitat</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tedania ignis</td>
<td>0.144 ± 0.29</td>
<td>0.76 ± 0.21</td>
<td>Present</td>
<td>1</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mycale microsigmatosa</td>
<td>0.152 ± 0.14</td>
<td>1.11 ± 0.34</td>
<td>Present</td>
<td>2</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Callyspongia pallida</td>
<td>0.091</td>
<td>0.72</td>
<td>Present</td>
<td>3,10</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chelonaplysilla erecta</td>
<td>0.082 ± 0.024</td>
<td>0.41 ± 0.06</td>
<td>Uncertain</td>
<td>11</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Haliclona caerulea</td>
<td>0.183 ± 0.171</td>
<td>0.73 ± 0.26</td>
<td>Present</td>
<td>4</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dysidea etheria</td>
<td>0.051</td>
<td>0.39</td>
<td>Present</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Desmapsamma anchorata</td>
<td>0.139 ± 0.041</td>
<td>0.81 ± 0.49</td>
<td>Present</td>
<td>6</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ircinia strobilina</td>
<td>0.950 ± 0.79</td>
<td>0.48 ± 0.12</td>
<td>Present</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Scopalina reutzleri</td>
<td>0.002</td>
<td>0.08</td>
<td>Absent</td>
<td>5</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aplysina archeri</td>
<td>0.007 ± 0.03</td>
<td>0.12 ± 0.13</td>
<td>Present</td>
<td>7</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cribrochalina vasculum</td>
<td>0.039</td>
<td>0.25</td>
<td>Present</td>
<td>8</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aiolochroia crassa</td>
<td>0.008</td>
<td>0.22</td>
<td>Present</td>
<td>3</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pandaros acanthifolium</td>
<td>0.029</td>
<td>0.08</td>
<td>Present</td>
<td>9</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Callyspongia vaginalis</td>
<td>0.007</td>
<td>0.03</td>
<td>Present</td>
<td>3</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Species (number of samples)  Sampling habitat

Abbreviations: M, Mangrove roots; R, Reef

a Abbreviations: G, Generalist species, occurring in mangrove habitats and adjacent reefs; R, Reef species

( - ) indicates no color development occurred, and (+,++,+++) represents an increase in color intensity

Observed directly with electron microscopy or inferred from numerical abundance in sponge extracts or fatty acid profiles. References: 1. Stierle et al. (1988); 2. Degoeij et al. (2008b); 3. Weisz et al. (2008); 4. Maldonado (2007); 5. Rützler et al. (2003); 6. Carballeira and Shalabi (1994); 7. Lee et al. (2001); 8. Carballeira and Reyes (1990); 9. Schmitz et al. (1981); 10. Uncertain for this particular species, but members of this genus contain bacteria; 11. Very few reports provide anecdotal evidence for the presence of bacteria.
(Cladochalina) vaginalis (Lamarck, 1814), were collected from the reef and are very common on shallow reef systems throughout the Caribbean, but are absent from mangrove habitats (Voss, 1976; Van Soest, 1978, 1980, 1984; Rützler et al, 2009).

Tannase activities of sponge species and their corresponding sampling and natural habitat are presented in table 1. Sponge species are divided in 2 groups based on their natural occurrence: generalist species, occurring in both mangrove and reef environments; and reef species, occurring in reef environments, but absent in mangrove environments. Color development was variable among specimens collected from the reef and mangrove roots. Except for S. Ruetzleri, tannase activity was present in all generalist species collected from both mangrove roots and the reef. All the presented generalist species have also been regularly observed in Caribbean mangrove systems. It should be noted, however, that D. anchorata appears more common in mangrove systems in Curacao (Keunen and Debrot, 1995; Hunting et al., 2008) compared to other mangrove sites in the Caribbean (e.g. Rützler et al, 2000). The species collected from both the reef and mangrove roots, i.e. D. anchorata and I. Strobilina showed comparable color development, irrespective of sampling habitat. In contrast, color development was clearly absent in sponge species that seem restricted to reef environments.

Sponge endobionts were inoculated on substrate containing mangrove extracts. Microscopic examination of the developed biofilm revealed that bacterial cells were more numerous, morphologically diverse, and motile in treatments containing sponge endobionts that naturally occur in mangrove habitats compared to endobiotic communities retrieved from sponge species typical of reef environments (data not presented). Apart from bacterial cells, we were unable to detect fungal hyphae, thereby we suspect that tannase activity and remineralization of mangrove-derived DOM in our incubations had a bacterial origin. We did not examine the taxonomic identities of the bacteria and whether these bacteria were actual symbionts of the investigated sponges (e.g. Transmission Electron Microscopy), and therefore we can not exclude the possibility that bacteria resided outside sponge tissue. In addition, the presented enzymatic activities may be an overestimation of actual activities due to the relatively long periods of incubation (24 h and 72 h). Current knowledge on sponge-microbe associations remains fragmented (Taylor et al., 2007), and previous studies on sponge-microbe symbiosis of sponge species included in this study often fail to provide evidence that bacteria are present within sponge tissue. Although information on bacterial presence in tissues of the collected sponges retrieved from primary literature (provided in table 1) suggests that, except for S. Reutzleri, all sponges appear to have
associations with bacteria, future work should resolve whether these bacteria are true symbionts.

The ability of the sponge microbial community to grow on substrates containing mangrove-derived DOM and the inherent degradation rates of mangrove extracts as approximated by DIC accumulation and ETSA are presented in table 1. The results suggest that sponges commonly associated with mangrove roots contain bacteria that are capable of degrading tannins, while bacterial communities associated with sponges that are more typical to reef environments appear less proficient in degrading mangrove tannins. Mangrove leachate is a complex mixture of compounds that are relatively resistant to microbial degradation and degradation of tannins is restricted to several bacterial and fungal species. Sponges attached to mangrove roots are in the direct vicinity of root leachates and exposed to high concentrations of tannins if tannins are allowed to accumulate. The presence of tannin-degrading symbionts may provide typical mangrove sponges a competitive advantage, provided that symbionts play an important role in the assimilation of organic matter (Weisz et al., 2008; de Goeij et al., 2008a). It has been demonstrated that typical mangrove species grow more rapidly on mangrove roots compared to typical reef species (Wulff, 2005). This growth rate advantage resulted in overgrowth of reef species by mangrove species, which eventually eliminated reef species from the mangrove environment (Wulff, 2005). Future research efforts should elucidate whether differences exist between sponge species from different natural habitats with respect to organic matter assimilation in order to validate the ecological relevancy of the presented observations.

A number of biotic and abiotic variables are considered important in mangrove-sponge endemism and community dynamics. The principal variables include current, temperature, salinity, tidal ranges, storm events, water quality, competition, predation and proximity to source populations (Wulff, 2005; Pawlik et al., 2007; Hunting et al., 2008), in which physico-chemical parameters become progressively variable with increasing distance from the equator. Our results suggest that the structure of the endobiontic community of sponges may contribute to the structural differences between mangrove and reef ecosystems. Although knowledge on the degree of host specificity in sponges remains fragmented, evidence exist that the structure of the endobiont communities is at least partly host specific in the majority of sponge species (Taylor et al., 2007: and references therein). Moreover, host specificity was shown to be important in sponge performance under differing physico-chemical conditions (e.g. Roberts et al., 2006). Host specificity of bacterial endobionts capable of degrading mangrove derived DOM and the presence of high concentrations of recalcitrant organic compounds in mangrove habitats
may improve competitive abilities of mangrove sponges and therefore lead
to ecological separation between mangrove and reef communities.
Chapter 10

Substrate as a driver of sponge distributions in mangrove ecosystems

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Abstract: Caribbean mangrove-associated sponge communities are very distinct from sponge communities living on nearby reefs, but the mechanisms that underlie this distinction remain uncertain. This study aimed to elucidate the relative importance of substrate and habitat in the ability of sponges to persist in mangrove ecosystems, and to evaluate the role of bacterial symbiont composition and carbon uptake in sponge distribution. Two reef species (Aplysina archeri and Desmapsamma anchorata) were transplanted to mangrove roots and PVC tubes at a mangrove stand and a reef site. The mangrove species Mycale microsigmatosa was transplanted to both substrates in mangroves as control, showing complete survival. In contrast, reduced survival was observed for D. anchorata with lowest survival on roots in mangroves, intermediate survival on both PVC in mangroves and roots on the reef, and complete survival on PVC on the reef. A. archeri had reduced survival in all treatments, but was most affected by the root substrate in mangroves. These results reveal that the inability of typical reef species to survive in mangrove ecosystems is related to habitat and substrate. The symbiotic bacterial communities were host specific and very similar before and after transplantation. The metabolic diversity of bacterial communities in A. archeri, M. microsigmatosa and D. anchorata strongly separated between host species and the surrounding water. It is speculated that compositional differences in dissolved organic matter (DOM) composition and symbiotic bacteria are potentially important in structuring sponge communities, explaining the exclusion of typical reef species and persistence of mangrove species in mangrove ecosystems.

Keywords: Dissolved organic matter, Sponges, Bacterial symbionts, Reef, Mangrove roots.
Submerged roots of mangroves along (sub-) tropical Caribbean coasts serve as a substrate for a diverse and dense sponge community. It is well documented that the species composition of these mangrove-associated sponge communities is very distinct from sponge communities living on nearby reefs (e.g. Van Soest 1978, 1980, 1984, Wulff 2004, Diaz et al. 2012), but the mechanisms that underlie this distinction remain uncertain (for review see: Wulff 2012). Transplantation experiments of typical reef sponges to roots on off-shore mangrove stands embedded in coral reefs revealed that reef species were able to grow well on roots and compete with typical mangrove sponges in the presence of spongivorous fishes (Wulff 2005). This suggests a major role for biological interactions steering sponge species composition. In contrast, typical reef species deteriorated quickly after transplantation to coastal mangroves in the absence of predation (Farnsworth & Ellison 1996, Wulff 2004, Pawlik et al. 2007), suggesting that abiotic factors are also important for sponge survival and perseverance in mangroves ecosystems, although it remained uncertain which abiotic factor was the key controlling variable.

Previous studies indicated that mangrove sponge community assembly mainly relies on small scale (i.e. among root scale) processes (Guerra Castro et al. 2011), which might be directly related to the root substrate (Hunting et al. 2010a,b). Mangrove-derived organic matter leaching from the roots and decomposing litter are the primary carbon sources for sponges living in mangrove habitats (Granek et al. 2009), and bacterial symbionts play an important role in assimilating these organic carbon sources (de Goeij et al. 2008a,b, Ribes et al. 2012). However, mangrove-derived dissolved organic matter (DOM) consists mainly of tannins and polyphenolic compounds (Maie & Jaffe 2006), which are structurally complex and recalcitrant to biodegradation (Field & Lettinga 1992, Koch et al. 2005, Kristensen et al. 2008). Increasing evidence suggests that only a limited number of bacterial and fungal species are able to degrade complex polyphenols and tannins (Bhat et al. 1998), and it has indeed been demonstrated that sponges commonly associated with mangrove roots contain bacteria that are capable of degrading mangrove-derived DOM, while bacterial communities associated with sponges that are typical to reef environments appear less proficient in degrading mangrove-derived DOM (Hunting et al. 2010b). It is therefore hypothesized that the presence of bacterial endosymbionts that are capable of degrading mangrove-derived DOM may explain the observed differences in species composition between mangrove and reef sponge communities.

Evaluating the role of habitat, substrate and symbiotic bacteria in driving sponge distributions thus requires experiments that discriminate effects of substrate and habitat (i.e. surrounding water) on the survival and perseverance of sponges in mangrove ecosystems, and assess symbiotic
bacterial community composition and metabolic diversity upon transplantation. We therefore 1) monitored survival and condition of typical mangrove and reef sponge species after in situ reciprocal transplantation to DOM-releasing mangrove roots and DOM-free surrogate roots (PVC tubes) in both mangrove and reef environments; 2) determined the structure and stability of the symbiotic bacterial community in the sponge host before and after transplantation; and 3) evaluated carbon utilization patterns of the symbiontic bacterial communities of the individual sponge species.

Methods

Study site

For this study, a location was chosen where reef and mangrove ecosystems were closely connected. Therefore, experiments were performed at the “Spaanse water” [N12°4”14.5’, W68°51”36.8’], and “Caracasbaai” [N12°4”11.4’, W68°51”43.8’], on the island of Curaçao, Netherlands Antilles, Caribbean Sea (Fig. 1). The inner bay “Spaanse Water” is connected to the open sea by a small channal, and monopolized by the red mangrove *Rhizophora mangle*. Sponges are the dominant epibionts on fringing roots (on average >10% coverage per root (Hunting et al. 2008)). Tidal ranges are approximately around 10 cm, which does not cause emergence of the resident sponge community. Further details on physicochemical characterization are provided elsewhere (Hunting et al. 2008). “Caracasbaai” is an adjacent reef dominated by corals and sponges. Both sites were used for sponge collection and as transplantation sites.

Fig. 1 Map of study area; 1) “Spaanse Water” (mangrove site) and 2) “Caracasbaai” (reef site) on Curaçao. Inset: shaded areas indicate study area.
Reciprocal transplantation experiment

Three sponge species were selected to investigate substrate and habitat effects upon transplantation in April and July 2010. 1) *Mycale microsigmatosa* inhabits mainly mangrove habitats and is a dominant species in mangroves in the Western Atlantic (Hunting et al. 2008, Wulff 2009). 2) *Desmapsamma anchorata* is an opportunistic species that abounds on reefs, but sometimes also occurs in mangrove habitats (McLean and Yoshioka 2008). 3) *Aplysina archeri*, a vasiform species, is commonly found on Caribbean reefs but is absent in mangroves, and therefore chosen to represent a typical reef species. Fragments of *M. microsigmatosa* (2-3 cm$^2$) were collected from mangrove roots and adjacent substrata in “Spaanse Water” at depths ranging from 0.9-2.3 m. Fragments of *A. archeri* (6-10 cm$^3$) and *D. anchorata* (2-6 cm$^3$) were collected from the “Caracasbaai” reef at depths ranging from 9.8-22.3 m and 4.8-8 m, respectively. Sponge fragments were transported in 25 L containers with natural seawater.

To distinguish between possible substrate and habitat effects, we used freshly cut prop roots of *R. mangle* and PVC tubes (Ø 40mm) as surrogate roots. PVC tubes were placed at both the mangrove site and reef site approximately one week before the start of the experiment to allow development of a biofilm required for sponge attachment (Ellison et al. 1996). Cleaned prop roots were cut two days before the start of the experiment. All substrates were placed at 2 m depth in both habitats. Fragments of the reef sponges *A. archeri* (n=80 per treatment) and *D. anchorata* (n=80 per treatment) were subsequently transplanted to both cut mangrove roots and PVC tubes in both the mangrove site and reef site (total of 320 specimens). Fragments of *M. microsigmatosa* (n=80 per treatment) were transplanted to both cut mangrove roots and PVC tubes within the mangrove habitat as control to exclude potential effects of transplantation and cutting of the root substrate (total of 160 specimens), although this does not rule out potential negative effects of root cutting on reef sponges. Plastic cable ties were used for attachment, which did not affect the sponges during the experiment. Survival was visually inspected after 0, 2, 4, 6, 8, 10, 14, 18, 24, 32 and 42 days. Confidence limits (CL95%) of the obtained proportions were approximated following Newcombe (1998).

*D. anchorata* is known for its unusual fast growth (Wulff 2005) and therefore an additional reciprocal transplantation experiment was performed to monitor changes in sponge tissue after transplantation of *D. anchorata* (n=20 per treatment) to mangrove roots and PVC-tubes in both the mangrove and reef habitat. Transplants were photographically monitored (Nikon digital underwater camera) on five occasions during two weeks, and images were evaluated for survival, necrosis (formation of
white patches), and development of new oscula. Confidence limits (CL95%) of the obtained proportions were approximated following Newcombe (1998).

**Bacterial community structure**

Subsamples were taken from the transplanted sponges to detect changes in bacterial community composition upon transplantation. Triplicate samples of all treatments were sampled immediately upon transplantation, and 25 days after initial sampling for *M. microsigmatosa*. Triplicates were sampled for *A. archeri, D. anchorata* at the point that >60% of the reef sponge transplants died (50 and 29 days after initial, respectively). Subsamples were taken from the internal healthy tissue (without noticeable necrosis) and stored in 100% Dimethylsulfoxide (DMSO) as described by Dawson et al. (1998). Bacterial DNA of the sponge-bacterial consortia was isolated as described by Hardoim et al. (2009). In brief, sponges stored in 100% DMSO were washed in 6.5 mL autoclaved water for 15 to 90 minutes and ground under liquid nitrogen to disrupt the sponge tissue and endoskeleton (mesohyl, mineral spicules and spongin fibers). DNA of ground samples was subsequently isolated using the UltraClean® Microbial DNA Isolation Kit (MO BIO, Carlsbad, New Mexico, US). An additional seawater sample (50mL) at both sites was sampled as control. DNA from filtered seawater samples (0.2μm Cellulose nitrate filter, Whatman®; NC 20) was isolated using PowerWater® DNA Isolation Kit (MO BIO, Carlsbad, New Mexico, US). Isolated DNA was amplified using the general bacterial forward primer F357 (GC CGC CCG CCG CCC GCG GGG CGG GGC GGG GGC CCT ACG GGA GGC AGC AG), containing a GC clamp (CGC CCG CCG CCC GCG CCC GGC CCG CCC GCG CCC C) at the 5' end, and reverse primer R518 (ATT ACC GCG GCT GCT GG) (Muyzer et al. 1993), amplifying the variable V3 region of the 16S ribosomal RNA gene using the following conditions: Initial denaturation: 94°C, 5 min. Cycling steps: 94°C, 30 sec., 54°C, 30 sec., 72°C, 1 min.; 35 cycles; Final elongation 72°C, 8 min.; Cooling 10°C for 15 min. on a MJ Research PTC-200 Thermo Cycler™ (St. Bruno, Quebec, Canada). Denaturing Gradient Gel-Electrophorese (DGGE) was performed on an Bio-Rad DCode™ system using 1 mm thick gels consisting of 8% (w/v) polyacrylamide (37,5 : 1 acrylamide : bisacrylamide) with a linear denaturing gradient from 30% to 55%, where 100% denaturing solution contained 7 M Urea and 40% (v/v) deionized formamide. The gels were run in 1x TAE buffer (Tris, Acidic acid and EDTA) at 60°C for 4 hours at 200 V and stained with Ethidium Bromide. Images of banding patterns were subsequently analyzed with Gelcompar II (Applied Maths, Kortrijk, Belgium), in which banding patterns of individual species were correlated by means of the unweighted pair group method with arithmetic mean.
(UPGMA) and Pearson’s correlation coefficients, and differences between sponge species were tested by a Mann-Whitney U test, where the within-group similarities were tested against the between-group similarities for each combination of species.

Metabolic diversity of sponge bacterial symbionts

In order to detect differences in C-resource utilization of the bacterial symbionts of the targeted sponge species, triplicate samples of *Mycale microsigmatosa*, *Desmapsamma anchorata* and *Aplysina archeri* were additionally collected in January 2013 and stored at 4°C for 1 week until analysis. Triplicate samples of the water of both the mangrove site (Spaanse Water) and the reef site (Caracasbaai) were included as control. Sponge samples were ethanol (70%) rinsed (30s) to reduce the potential contribution of superficial bacteria in the analysis. Bacterial endosymbionts of the sponge specimens were extracted from subsamples of sponge tissue (0.1 cm3) in a Precellys® 24 lysis/homogenizer (Bertin Technologies, France) using Ø 0.1 and 0.5-mm beads and subsequent centrifugation for

![Fig. 2](image_url) Survival percentages of A. archeri (n=320, t=42 d) and D. anchorata (n=320, t=15 d) after transplantation from the reef site to mangrove roots and PVC tubes at the reef and mangrove site, and M. microsigmatosa (n=160, t=57 d) after transplantation within the mangrove site to mangrove roots and PVC tubes. Error bars indicate approximated 95% confidence limits (C.L.). Bars sharing the same letters are not significantly different at the p < 0.05 level.
30 s at 11,000 g (Hunting et al. 2010b). We assessed bacterial metabolic diversity by community level physiological profiling (CLPP) using Biolog® GN microplates containing 95 unique single substrates (Biolog, Inc., Hayward, USA) (Garland and Mills 1991, Garland 1997). The 95 substrates in the Biolog GN plate are comprised of simple, common substrates (e.g. Sucrose, Mallose and Citric Acid), selected on their ability to discriminate among bacterial isolates (Bochner 1989). Biolog GN plates do not include recalcitrant substrates or substrates typical of mangrove DOM, and therefore this approach can only be used to illustrate that microbial communities are functionally similar or distinct (Garland 1999). Samples were incubated for 48h and utilization patterns of 95 different single carbon sources were analyzed using a Jaccard-based cluster analysis and one-way ANOSIM and a bonferroni corrected pair wise comparison (Hammer et al. 2001).

**Results**

*Survival and condition of sponge species*

In the control treatments, almost all specimens (>90%) of the sponge *M. microsigmatosa* survived transplantation to mangrove roots and PVC tubes in the mangrove system (Fig. 2), with no significant (p>0.05) difference observed in survival between treatments. In contrast, only a small proportion (17%) of transplants of the sponge *A. archeri* survived transplantation to mangrove roots in the mangrove habitat, which was twice as low compared to transplants to mangrove roots and PVC tubes on the reef or onto PVC tubes in mangroves, (all 57% survival) (Fig. 2). The poor survival of *A. archeri* in all treatments (max 57%) indicates that this species was negatively affected by the transplantation. The lowest survival (17%) observed on mangrove roots in the mangrove site suggests that only substrate effected the survival of *A. archeri*. Transplants of the opportunistic sponge *D. anchorata* performed better compared to transplants of *A. archeri*. *D. anchorata* showed a more gradual mortality compared to *A. archeri*, with lowest survival on roots in mangroves, intermediate survival on PVC tubes in mangroves and roots on the reef (Fig. 2), and complete survival was observed for specimens transplanted to PVC tubes at the reef site. These results indicate a combined effect of substrate and habitat on the survival of *D. anchorata*.

In the photographic monitoring experiment, clear differences were also visible in the development of *D. anchorata* after transplantation, indicated by survival, necrosis and oscula formation. Representative photographs of the most dominant changes per treatment are shown in figure 3, and proportions are presented in figure 4. In the mangrove-root, mangrove-PVC and reef-root treatments, necrosis was already clearly
Fig. 3: Photographic recordings of the development of the sponge *D. anchorata* after transplantation from the reef site to mangrove roots and PVC tubes at the reef site and the mangrove site. Photographs were taken on day 0, 4 and 15. The photographs are representative of all replicates for each treatment.
visible on day 4, eventually resulting in total mortality in mangrove-root transplants and partial survival in specimens transplanted to mangrove-pvc and reef-root. The stress caused by the mangrove habitat and/or mangrove root was visible as tissue degradation and alteration. In contrast, all specimens transplanted to PVC at the reef site appeared healthy and formed a number of new oscula at the end of the experiment (day 15), which was also the case for the majority of specimens (~60%) that survived transplantation to mangrove roots at the reef site. Sponge condition generally reflected the same ranking as shown in Fig. 2, i.e. the worst condition was observed on roots in mangroves, intermediate conditions on PVC tubes in mangroves and roots on the reef (Fig. 2), while all specimens transplanted to PVC tubes at the reef site performed very well.

**Bacterial community structure and metabolic diversity**

Cluster analysis of the symbiotic bacterial community composition for replicates (n=3) of the three sponge species (*M. microsigmatosa*; *D. anchorata* and *A. archeri*) and their surrounding waters revealed that the bacterial community composition depended largely on host species (for all species: Mann-Whitney U; p < 0.001), irrespective of sampling time and habitat (Fig. 5). Samples of the surrounding waters of the reef and mangrove ecosystem also clustered together and were distinct from sponge endosymbiotic communities (Fig. 5). DGGE patterns of individual sponge species are provided as supplementary material (Fig. S1-3).

![Fig. 4: Condition of the sponge D. anchorata after transplantation from the reef site to mangrove roots and PVC tubes in the mangrove site and the reef site. Condition is categorized by mortality (after 15 d), necrosis (during experiment), and oscula development (after 15 d) as percentage of total number of transplants. Error bars indicate approximated 95% confidence limits (C.L.). Bars sharing the same letters are not significantly different at the p < 0.05 level.](image-url)
Bacterial communities derived from the three sponge species shared a number of carbon sources, but also used a number of unique carbon sources (Supplementary table 1), including e.g. Glycerol and 2-Amino-Ethanol (A. archeri), D,L-Camitine and Succinic acid (D. anchorata), and Phenylethylamine and Hydroxy-L-proline (M. microsigmatosa). Consequently, cluster analysis of metabolic diversity of bacterial communities in the sponge species A. archeri, M. microsigmatosa and D. anchorata, and the control water samples revealed a strong separation between sponge host species as well as the water samples (Fig. 6) (ANOSIM, bonferroni corrected pair wise comparison: p<0.05).

![Pearson's based cluster analysis of sponge-associated bacterial community composition](image)

*Fig. 5: Pearson's based cluster analysis of sponge-associated bacterial community composition of samples of M. microsigmatosa, D. anchorata and A. archeri before and after transplantation from the reef site to mangrove roots and PVC tubes at the reef site and the mangrove site. Microbial community composition was significantly different between species (for all species: Mann-Whitney U; p < 0.001).*
Discussion

Transplantation of the typical reef sponge *A. archeri* and the opportunistic sponge *D. anchorata* on roots in both the reef and mangrove ecosystem revealed high mortality. This result is in line with previous transplantation experiments where typical reef species were similarly transplanted to mangrove roots, in which potential effects of competition were excluded (Ellison & Farnsworth 1992, Pawlik et al. 2007). Photographic recordings of *D. anchorata* revealed substantial necrosis and mortality in all mangrove and root related treatments, while specimens transplanted to PVC-tubes on the reef developed very well. This outcome illustrates that, in addition to biotic interactions like predation and competition for space (Wulff 2005) and extreme fluctuations in abiotic conditions (Pawlik et al. 2007), root substrate is also critically important in limiting survival of typical reef species in mangrove systems. A large part (~50%) of the *A. archeri* transplants did not survive transplantation in all treatments. Since *A. archeri* typically occurs much deeper than the depth used in our study, it is possible that *A. archeri* transplants were adversely affected by e.g. stressful light intensities. Despite this, survival of *A. archeri* was clearly reduced when transplanted to roots in mangroves, thereby suggesting a substrate effect. *D. anchorata* developed well on PVC tubes on the reef, but survival of *D. anchorata* was reduced when transplanted.
to roots on the reefs site and PVC in mangroves, demonstrating that besides the root substrate, the surrounding water or habitat in general also affected *D. anchorata* in mangrove ecosystems. Both substrate and abiotic constraints thus affected survival and hampered perseverance of typical reef sponges in mangrove ecosystems.

The question remains why typical mangrove sponge species such as *Mycale microsigmatosa* are capable of maintaining viable populations on mangrove roots in coastal mangrove ecosystems, and why typical reef species are negatively affected by the root substrate. We hypothesize that bacterial symbionts play an important role. Although there is a growing amount of literature on community composition of sponge endosymbionts, little is known about shifts in these communities induced by biotic and abiotic factors and over time (Thacker & Freeman 2012). Although we only sampled two time points, profiling bacterial communities by means of DGGE suggested that the symbiotic communities in the sponges remained mainly host specific, irrespective of time and treatment (substrate and habitat). This is in line with other studies that considered symbiotic bacterial communities over larger temporal scales (e.g. Hentschel et al. 2002, Yang et al. 2011, Erwin et al., 2012).

Our study further revealed that the host specific bacterial symbionts used different carbon sources, suggesting that each sponge symbiotic bacterial community has its own specific resource niche, as frequently observed for free living environmental bacterial communities (e.g. Salles et al. 2009, Gravel et al. 2011). However, the use of Biolog plates comes with limitations, as ecological relevant substrates are not captured in this assay, and therefore we can not directly relate substrate utilization profiles to distribution patterns of sponge-bacterial consortia. Nonetheless, we speculate that differences in resource niches could potentially be important, especially considering that DOM, although not characterized in this study, often differs both in composition and concentration between mangrove and reef habitats (e.g. Dittmar et al. 2006). It has been demonstrated that the variation in bacterial communities within sponges coincide with metabolic differences between sponge species (Weisz et al. 2007), and that rates of Dissolved Organic Carbon (DOC) and nutrient uptake by sponges largely depend on the concentration of symbiotic bacteria (Ribes et al. 2012). Evidence for sponge resource preferences is also evident from studies that considered isotope ratios. For instance, Van Duyl et al. (2012) showed that encrusting sponges in reef cavities feed mainly on DOM derived from crustose coralline algae and coral mucus, while mangrove-derived organic matter is an important C-source for sponges that typically occur in mangrove ecosystems (Granek et al. 2009). Although the utilization profiles of simple substrates obtained in this study do not directly relate to mangrove DOM, they complement a previous
study that reported differences in the ability of bacterial symbionts to degrade mangrove DOM (Hunting et al. 2010b). Bacterial symbionts of mangrove sponges appeared proficient in degrading mangrove DOM, while symbionts of reef sponges were less capable of degrading mangrove DOM. Earlier studies also showed that common mangrove-associated sponges grew faster when attached to mangrove roots compared to PVC tubes, while typical reef species performed better on PVC than on the root substrate (Ellison et al. 1996, Wulff 2005). Although this remain speculative and requires further investigation, this suggests that typical mangrove species potentially have a competitive advantage over reef species when growing on mangrove roots, and that the composition of DOM can be of general importance to the performance of sponge-bacterial consortia. However, since mangrove DOM consists of a complex mixture of structurally diverse compounds, a major challenge remains the identification of specific bacterial gene-clusters (e.g. pyrosequencing) and relevant compounds in DOM (e.g. gas chromatography – mass spectrometry, GC-MS), and ultimately how these components interact and relate to the overall performance of sponge-bacterial associations under natural conditions.

In conclusion, increased mortality and poor development upon reciprocal transplantation revealed that the inability of typical reef species to survive in mangrove ecosystems is due to a combined effect of abiotic constraints and the root substrate. Our results further suggested that bacterial symbionts are largely host specific and have a specific DOM resource niche, hinting that differences in DOM composition and corresponding differences in symbiotic bacterial communities potentially are important in structuring sponge community composition, which would explain the exclusion of typical reef species and the persistence of mangrove species in mangrove ecosystems.

1 Supplementary data related to this chapter can be found at: www.int-res.com/articles/suppl/ m486p133 supp.pdf
Chapter 11

Root-derived organic matter confines sponge diversity in mangrove ecosystems

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Abstract: Caribbean mangrove-associated sponge communities are very distinct from sponge communities living on nearby reefs, but the mechanisms that underlie this distinction remain uncertain. It has been hypothesized that dissolved organic matter (DOM) leaching from mangrove roots, and the ability of mangrove-associated sponge-bacterial consortia to degrade mangrove-DOM, may cause this distinction. This study tested whether mangrove-DOM, leaching from mimicry substrates or directly injected in sponge tissue, affected the performance of a reef and a mangrove sponge species. Controls and the mangrove sponge remained unaffected by mangrove-DOM leaching from mimicry substrates or directly injected in sponge tissue, but the reef species showed substantial necrosis when exposed to mangrove-DOM. Results presented in this study suggest that mangrove-DOM confines the composition of sponge communities in mangrove ecosystems, explaining the exclusion of typical reef species and the adjacent occurrence of distinct sponge communities.

Keywords: Dissolved organic matter, Mangrove root, Necrosis, Polyphenols, Sponges, Reef.
Submerged roots of mangroves along (sub-) tropical Caribbean coasts serve as a substrate that is dominated by sponges. Mangrove-associated sponge communities are relatively species poor and distinct from the diverse sponge communities living on nearby reefs (e.g. Van Soest 1978, 1980, 1984, Wulff 2004, Diaz et al. 2012). However, the mechanisms that underlie this distinction remain uncertain (for review see: Wulff 2012). Transplantation experiments of typical reef sponges to mangrove roots revealed that reef species deteriorated quickly after transplantation to coastal mangrove roots (Farnsworth & Ellison 1996, Wulff 2004, Pawlik et al. 2007), in which the root substrate is critically important in limiting survival of reef species in mangrove systems (Hunting et al., 2013), while mangrove species remain unaffected. However, the question remains what causes the typically observed deterioration of reef species.

Dissolved organic matter (DOM) is a primary food source for sponge-bacterial consortia (de Goeij et al. 2008a,b), and it has been hypothesized that DOM leaching from mangrove roots plays an important role in structuring mangrove sponge community composition (Hunting et al 2010b). Mangrove-DOM consists mainly of tannins and polyphenolic compounds (Maie & Jaffe 2006), which are structurally complex and recalcitrant to biodegradation (Field & Lettinga 1992, Koch et al. 2005, Kristensen et al. 2008), thereby reducing mangrove palatability and inhibiting growth of fouling organisms on mangrove roots (e.g., Schmitt et al. 1998). A limited number of bacterial and fungal species are able to degrade complex polyphenols and tannins (Bhat et al. 1998). Therefore, the ability of mangrove associated sponge-bacterial consortia to degrade mangrove-DOM may explain the persistence of mangrove species in mangrove ecosystems, while the inability to degrade mangrove-DOM could potentially hamper fouling of roots by reef species. However, this remains to be proven experimentally, and therefore this study aimed to test whether mangrove-DOM leachates from roots are responsible for the observed deterioration of reef species transplanted to mangrove roots. To this end, we (1) transplanted a typical reef species and a typical mangrove species to mimicry substrates containing mangrove root extract and to control substrates without extract; and (2) injected mangrove DOM directly into tissues of both sponge species.

Methods

Study site and sponge collection

This study was conducted in Curaçao, N.A., southern Caribbean, in February and March 2013. The selected species included the mangrove sponge *Teda nia ignis*, collected from the inner bays Spaanse Water and Piscaderbaai, and the reef sponge *Desmapsamma anchorata*, collected at the site at which the transplantation experiment was performed: the
shallow reefs in front of the research facility of Carmabi (Caribbean Research and Management of Biodiversity). Both species were chosen for their fast growth (Wulff, 2005; Wulff, 2012). For detailed maps of the study sites, see Hunting et al. (2008, 2010a) and De Goeij et al. (2008b).

**Transplantation experiment**

The effect of mangrove DOM on sponges was assessed with artificial substrates made from agar that allows mangrove root extracts to slowly diffuse into the overlying water (e.g., Henrikson and Pawlik 1995; Browne and Zimmer 2001; Hunting et al., 2010a). Each mimicry gel was prepared in plastic containers (Volume 100 cm$^3$) and consisted of 3% agar (Molecular Genetics/Granulated, Roth, Germany), and 1 mL of mangrove root extract, added to the agar suspension at ~50°C. Mangrove DOM was extracted from freeze-dried and ground *R. mangle* roots (40 g dry weight) with 70% aqueous acetone for 48 hrs. Root material was subsequently discarded, and the extract containing acetone was centrifuged (4000 rpm 15 min) and air-dried in a flow cabinet. The remaining pellet was subsequently resuspended in 100 mL deionized water. Gels were covered with plankton net (mesh size 2.0 mm) to facilitate attachment of sponge transplants with plastic cable ties (Ellison et al. 1996). Five specimens of both *T. ignis* and *D. anchorata* (2-3 cm$^3$) were transplanted to mangrove DOM containing gels, while an additional five specimens of both species were transplanted to gels without mangrove DOM as control. Transplants were evaluated for percentage tissue necrosis (formation of white and black lesions) after 1 week and compared with a 2-sample z-test.

**Injection experiment**

An additional experiment was performed to assess the direct effect of mangrove DOM on sponges by injecting mangrove DOM in sponge tissues. Five specimens of both *T. ignis* and *D. anchorata* were *in situ* injected with dilute (1:50) mangrove extract, and an additional five specimens of both species were injected with seawater as control. Injected sponges were evaluated for percentage tissue necrosis (formation of white and black lesions) after 1 week and compared with a 2-sample z-test.

**Results and Discussion**

All specimens of the typical reef species *D. anchorata* transplanted to control substrates without mangrove DOM developed very well (Fig. 1A). In contrast, 60% (3 out of 5) of the specimens transplanted to substrates containing mangrove DOM showed substantial (40-100% of the tissue) necrosis (white-colored lesions) after 1 week (Fig. 1D), suggesting that mangrove DOM significantly affected the performance of *D. anchorata*
Fig. 1: Photographic recordings of the development of the reef sponge D. anchorata after transplantation to control gels without mangrove root extract (A) and mimicry gels containing mangrove root extract (B); T. ignis after transplantation to control gels without mangrove root extract (C) and mimicry gels containing mangrove root extract (D); and direct effects of the injection of seawater (E) and dilute mangrove root extract (F) on D. anchorata. Photographs were taken on day 7. The photographs are representative for each treatment (n=5).
(Two sample z-test, \( z = 2.1, \ p = 0.038 \)). This response is in agreement with previous observations on reef sponges transplanted to mangrove roots (Farnsworth & Ellison 1996, Wulff 2004, Pawlik et al. 2007, Hunting et al. 2013). The mangrove sponge \( T. \ ignis \) did not reveal any sign of necrosis on substrates containing mangrove DOM, nor on the control substrates (Fig. 1B,D). Similarly, injection of dilute mangrove extract in \( D. \ anchorata \) resulted in necrosis in all specimens, primarily various degrees of black colored lesions surrounding the site of injection, and altered shape in 2 specimens, while controls injected with seawater remained unaffected (Fig. 1C,F). All \( T. \ ignis \) specimens remained unaffected by the injection of both dilute mangrove DOM and seawater (not shown). Injection of mangrove DOM also significantly affected the performance of \( D. \ anchorata \) (Two sample z-test, \( z=3.2, \ p=0.0016 \)).

The present study provided experimental evidence that mangrove-DOM can exert negative effects on reef sponges, while mangrove-associated sponges remain unaffected. It has been demonstrated that mangrove-derived organic matter is a major carbon source for sponges living in mangrove ecosystems (Granek et al. 2009), while reef species feed mainly on DOM derived from crustose coralline algae and coral mucus (Van Duyl ey al. 2012). Sponges attached to mangrove roots are in the direct vicinity of root leachates and exposed to high concentrations of DOM when DOM accumulates in the surrounding water. Our observations thus suggest that mangrove-DOM prevents typical reef species to thrive in mangrove ecosystems, and support the notion that the composition of DOM can be of general importance to the performance of sponge-bacterial consortia (Hunting et al., 2013).

This study aimed to test whether mangrove-DOM leachates from roots are responsible for the typically observed deterioration of reef species transplanted to mangrove roots. We observed that mangrove-DOM induced necrosis in a reef sponge, while a mangrove-associated sponge remained unaffected. The inability of reef species to cope with mangrove-DOM may therefore confine the composition of mangrove-associated sponge communities, explaining the exclusion of typical reef species and the adjacent occurrence of distinct sponge communities.
Chapter 12

Concluding remarks
This thesis identified processes linking biotic and abiotic components of two contrasting benthic detrital food webs on either soft bottom sediments or on hard mangrove root substrates. This concluding chapter evaluates the importance of biotic-abiotic interactions in these two habitats and attempts to identify general drivers for detritus processing in benthic detrital food webs.

Organic matter composition

Organic matter in natural ecosystems varies widely in chemical composition. Some components of detritus are readily utilized, while other components resist degradation (recalcitrant carbon) or inhibit microbial and invertebrate consumers (Gessner and Chauvet, 2002; Cornwell et al., 2008; Hladyz et al., 2009). This thesis demonstrated that organic matter composition drives decomposition in different natural and manipulated benthic habitats.

Firstly, mangrove-derived OM, originating from decaying leaves and leachates from mangrove roots, is mainly composed of tannins and polyphenolic compounds (Maie and Jaffe, 2006). These recalcitrant substrates were shown to enhance recruitment only of specific mangrove-associated sponge species, while other species were suppressed (Chapter 8). In addition, it was demonstrated that exposure to mangrove-derived OM can kill sponge species that do not naturally colonize on mangrove roots within few days (Chapter 11). The capacity of the sponge species to grow on mangrove roots appeared to concur with the capacity of their associated bacterial symbionts that appeared to benefit from degrading recalcitrant organic substrates (Chapter 9). Therefore, the sorting of sponge holobionts capable or incapable of colonizing mangrove roots is essentially caused by the chemical composition of mangrove-derived OM.

Secondly, in this thesis the development of freshwater bacterial communities was monitored on two contrasting substrates: freshly collected plant biomass (ground nettle) and recalcitrant peat. In the experiment reported in Chapter 5, the metabolization of common, easily degradable compounds was used as a proxy to detect differences in the functional characteristics that ideally would involve also the metabolization of more recalcitrant substrates such as aromatic compounds in peat. It was shown that the functional composition of bacterial communities develops differently depending on the substrate type (Chapter 5). These observations are consistent with substrate-dependent developments of bacterial communities in humic lakes (Eiler et al., 2003) and soils (Brant et al., 2006).

Thirdly, a decomposition and consumption tablet (DECOTAB) with a defined chemical composition was developed to gauge detritus processing rates in situ (Chapter 4). Two different DECOTABS were composed and
tested: a standard DECOTAB composed mainly of cellulose, and a more complex DECOTAB containing additional plant and soil extracts. It was demonstrated that microbial organic matter processing rates can differ depending on organic matter composition, while no differences in feeding rates were observed in the litter shredding isopod Asellus aquaticus. This indicates that organic matter composition has stronger and more direct effects on the microbial community than on the invertebrate communities in soft bottom sediments. Recently, litter decomposition rates in stream beds and forest floors have been compared in conjunction with consortia of invertebrates and microorganisms, and differences between both habitats were demonstrated (Gessner et al., 2010, and references therein). For instance, terrestrial detritivores accelerated decomposition of recalcitrant leaf species, while stream detritivores confronted with diverse litter fed preferentially on high-quality leaves, resulting in lowered OM processing rates. The observed difference in the effect of organic matter composition on invertebrates in soft sediments and mangrove root substrates suggests that different mechanisms underlie the effect organic matter composition on links between biota and OM processing rates. The present thesis demonstrates that organic matter composition invokes direct effects on either microbes or invertebrate-bacterial consortia, thereby affecting the development of specific invertebrate-bacteria consortia on large spatio-temporal scales. Thus the insight that organic matter composition is an important driver for OM processing rates highlights the need to study organic matter composition as driver for the long-term development of detritus based communities.

**Invertebrate-Bacteria-Substrate interactions**

Invertebrates and microorganisms are tied together in the processing of organic matter, and both groups of organisms can provide mutual benefits. This thesis also addressed these two sides of the same coin. This thesis demonstrated that sediment reworking by invertebrates strongly promote bacterial respiration and organic matter processing in aquatic sediments, in which the magnitude of bacterial activity and detritus processing relied heavily on the type invertebrate bioturbation (Chapter 2 and 3). Moreover, invertebrate bioturbation also influenced the structure of the bacterial community and the spatio-temporal redox (Eh) conditions within the sediment. Thereby sediment reworking by invertebrates strongly facilitated and promoted bacterial growth in (sub)surface sediments.

Positive feedback between fauna and bacteria is even stronger in sponges. Sponges provide a physical substrate for microorganisms that colonize sponge voids or are actually incorporated in sponge tissues, in which symbiotic microorganisms can make up a large part (> 50%) of the...
holobionts biomass (e.g. Taylor et al., 2007). Sponge hosts actively filter tremendous amount of particulate and dissolved OM and the microbial symbionts play a role in the transfer of carbon substrates to its sponge host (Freeman and Thacker, 2011; Ribes et al., 2012). However, exact metabolic pathways and exchange routes of organic substrates between sponge cells and bacterial symbionts remain completely unresolved to date (Thacker and Freeman, 2012). Results presented in this thesis show that specific sponge-bacterial consortia living on mangrove roots were capable of degrading recalcitrant organic matter leaching from mangrove roots (Chapter 9), while sponge-bacterial consortia occurring on reefs can not utilize mangrove-derived OM (Chapter 9), nor survive exposure to mangrove-derived OM (Chapter 10, 11). This indicates that sponge-bacterial consortia interact in (D)OM metabolism facilitating growth on mangrove roots and persistence in mangrove ecosystems.

The results presented in this thesis show that bacteria and invertebrates co-act in the process of OM degradation. Mutual benefits are evident in sediments that support free-living fauna and free-living microbes, but appears to be even stronger in sessile sponge-bacteria associations. These facilitative interactions potentially act via the joint accumulation and digestion of detritus (OM) by the faunal-bacterial consortia, yet the provision of a physical substrate and transport of OM, oxygen and other substrates by fauna is fundamental in both soft sediment and hard substrate communities.

**Anthropogenic stressors and organic matter processing**

Species-specific intolerance to abiotic constrains such as temperature, pH, salinity, or anthropogenic stressors like eutrophication and metal pollution, eventually limit the occurrence of aquatic organisms to certain ranges of natural environments (e.g. Kiffney et al., 1994; Bervoets et al., 1996). Thus, abiotic conditions may restrict the ecological processes sustained by these species. In particular, there is ample evidence for reduced detritus degradation in natural habitats. An obvious example is provided by peat-ecosystems that are characterized by the conservation of organic matter by water logging and acidic conditions (Freeman et al., 2001) and forest humic layer thickening (e.g. Jobbagy and Jackson, 2000). Thus, diversity losses induced by abiotic conditions can also results in deminished ecosystem functioning. For instance, at a small scale, the manipulated disturbance of populations of suspension-feeding caddisflies was observed to negatively affect organic matter fluxes in streams (Cardinale and Palmer, 2002) probably with downstream effects on aquatic communities. Therefore, interactions between selective abiotic conditions, biodiversity at different trophic levels, and overall process rates in detritivore communities are tightly linked, and an integrated approach is
essential to understand emerging patterns in interactions between biodiversity and ecosystem functioning. This thesis considered metal pollution as a potential driver influencing interactions between biota and organic matter processing and will be discussed below.

Effects of anthropogenic stressors are currently assessed by monitoring shifts and decreases in species composition of natural communities rather than subsequent changes in ecosystem processes. As a consequence, environmental policies aim at the protection of species, assuming inherent protection of ecosystem functioning. However, abiotic pressures may have various indirect effects via e.g. the reduction of food quality, invertebrate fitness or habitat suitability (e.g. Van der Geest et al., 1999; O’Halloran et al., 2008). This thesis therefore evaluated chemical and physical stress on invertebrate and bacterial detritivorous communities in relation to changes in ecosystem processes.

The response of a simplified soft bottom community to chemical stress was analyzed (Chapter 3). It was demonstrated that copper exposure affected the burrowing behavior of the isopod *Asellus aquaticus*, which resulted in reduced bacterial activity and organic matter processing; thereby illustrating that sub-lethal effects on invertebrates can cascade toward disordering ecosystem processes. Sub-lethal, physiological effects of pollution have also been observed in marine sponges, in which it was shown that OM filtration and clearance rates in sponges are reduced with increasing copper levels (Cebrian et al., 2006). Thus, anthropogenic stressors precede effects on biodiversity requiring a longer period of time to emerge, and therefore functional parameters (e.g. decomposition rate) may serve as more sensitive and reliable parameters for assessing ecological water quality and ecosystem functioning.

Metal pollution was thus found to exert strong effects on the performance of benthic communities and the inherent processing of organic matter. The profound impact of anthropogenic stressors on OM processing strengthen the insight that abiotic variables dictate organic matter processing both directly and indirectly and this warrants a key stone position in environmental protection aiming at protecting the species composition of communities, as well as protecting the essential functioning of ecosystems and the services they provide.

*Effects of solar radiation on benthic environments*

Solar radiation, and especially UV radiation, is known to exert negative effects on organisms and these effects depend strongly on the climate zone, climate changes and the transmission of radiation in local waters. Our current understanding of the effects of solar radiation is based on studies focusing on terrestrial systems and plankton communities, and it remained uncertain whether these observed effects extend towards the
benthic environment. Evidence is provided here that solar radiation is a currently overlooked, but important driver for benthic communities that can mask or even decouple invertebrate-bacterial interactions in detrital food webs. (Chapters 5 and 6). It is also shown that solar radiation at levels common in the temperate zone can indirectly affect benthic communities via most likely an altered organic matter composition and effects on microbial community composition (Chapter 5) as well as via altered redox conditions within soft bottom sediments (Chapter 6). A number of sponge species are also known to be negatively affected by increased radiation intensities in the tropics, and a reduced performance was observed in a reef sponges transplanted to shallow, experimental mangrove stands exposed more directly to solar radiation (Chapter 10). The detrimental effects in the tropics are currently attributed to stress responses of phototrophic symbionts (e.g. Diaz et al., 2012). However, DOM composition in coastal waters has been shown to change substantially due to solar radiation that induced photodegradation and photosensitization (Kattner et al., 2006; Dittmar et al., 2006), hinting that effects of solar radiation might also be more complex and seize upon different attributes of tropical benthic ecosystems. Hence, although the results presented in this thesis are based on short term incubations in simplified settings, they indicate that solar radiation is a currently neglected, but potentially profound abiotic variable influencing OM processing in benthic detrital food webs.

Organism traits and detritus processing

It is argued in Chapters 2, 3, 5, 6 and 10 that a functional characterization of communities, i.e. identifying invertebrate and bacterial species according to their traits that likely affect ecosystem processes (e.g. body size, feeding strategy, locomotion, substrate preference) is essential to understand overall ecosystem processes, in particular detritus processing. This argument accords with the meta-analyses by Gessner et al. (2010) and Stewart et al. (2013) of biodiversity effects on aquatic and terrestrial food webs, which stressed the paucity of observations on functional traits in detrital food webs. Following a similar consideration, Naeem and Bunker (2009) and Mulder et al. (2012) argued that the identification and quantification of functional traits relevant to ecosystem properties is a key issue that requires further assessment and consolidation. This thesis therefore considered functional traits of both invertebrates and microorganisms in organic matter processing.

In soft bottom sediments, this thesis demonstrated that the rate of organic matter processing was not related to taxonomic diversity of an aquatic invertebrate community, but directly related to the functional (bioturbation type) composition of the aquatic invertebrate community.
(Chapter 6), highlighting the importance of functional traits in soft bottom sediments (Nogaro et al., 2009; Hillebrand and Matthiessen, 2009). In contrast to the well studied invertebrate communities in soft bottom sediments, the functional role of sponges remains underexposed. Only a few seminal studies considered sponge functional traits, based mainly on body size and morphology, in relation to sponge species composition (Wulff, 2001; Bell, 2007, 2008; De Voogd and Cleary, 2007), yet consequences of shifts in sponge community composition for ecosystem functioning to date remain uncertain. However, sponges are of major importance for the processing of DOM and POM that is unavailable to metazoans (Bell, 2008; De Goeij et al., 2008; Thacker and Freeman, 2012), and transfer substantial amounts of dissolved carbon substrates in the form of particulate organic matter to higher trophic levels, thereby facilitating and sustaining biodiversity hotspots under oligotrophic conditions (De Goeij et al., submitted). Differential responses of sponges (Chapter 9, 10 and 11) and symbiont bacteria (Chapter 9) to mangrove-derived DOM and differences in OM resource preferences (cf. Granek et al., 2009; Van Duyl et al., 2012), however, point to an interplay between DOM composition and resource niches of sponge-bacterial consortia, and therefore DOM resource niches provide a promising functional trait linking sponge community composition to organic matter processing (Chapter 10).

Similarly, microorganisms can utilize a limited number of resources depending on physiological ability to metabolize organic substances. Bacterial communities likely respond to changes in the chemical composition of available organic matter, while alterations in bacterial community structure and functioning can become evident as the metabolic potential of the bacterial community studied. In this thesis, bacterial functional diversity was considered as bacterial resource niches, i.e. the type of substrates that are utilized by the consortium (e.g. Salles et al. 2009). This characterization proved useful to gain insight in the metabolic differentiation of sponge-associated bacterial symbionts (Chapter 9), and to describe changes in the functional composition of bacterial communities in response to various drivers such as including bioturbation (Chapter 3), organic matter quality (Chapter 5), and solar irradiation (Chapter 5). The results gained in this thesis thus demonstrated that the metabolic potential of bacterial communities provides valuable information about bacterial communities responsible for the processing of organic matter. This would have been overlooked when bacterial communities would have been characterized via their taxonomy, thereby supporting the notion that ecophysiological traits are valuable and worth extending to allow for an integrated assessment of bacterial taxonomic identity and metabolic capacity in the future.
Overall, this thesis identified functional traits for both bacteria and invertebrates. It was shown that trait diversity is more important than species diversity, thus demonstrating that functional traits are good vehicles to analyze the linkage between biodiversity and ecosystem processes.

**Conclusion**

This thesis identified interactions between fauna and micro-organisms degrading organic matter in two contrasting benthic systems: soft bottom sediments and hard mangrove root substrates. It was found that the chemical composition of OM and abiotic conditions, such as the presence of pollutants and solar radiation, greatly modulate biotic interactions.

Invertebrate fauna, free living in sediments and fauna attached to solid substrates (sponges) were found to facilitate the activity and composition of bacterial consortia, while vice versa bacterial development conditioned organic matter that was either refractive (peat) or less palatable (mangrove phenolics) for fauna. These refractory components of mangrove organic matter structured the sponge species distribution over mangrove and coral reef substrates. Biotic interactions appeared to be affected via faunal bioturbation modifying sediment redox conditions, or immobilization thereof by a model toxicant (Cu). Susceptibility of the detrital food web to copper has been demonstrated to interfere with detritus degradation, thereby demonstrating that effects of anthropogenic stressors on ecosystem processes precede effects on biodiversity. For that reason functional parameters for measuring ecosystem integrity are promoted. Solar radiation influenced the redox conditions of the sediment, thereby blurring the concurrent effects of bioturbation, and overriding the faunal effects on sediment bacteria. Although biodiversity or species composition has served as a touchstone for environmental protection, throughout the present study I found better explanations for detritus decay dynamics when using functional parameters of fauna and bacterial consortia.
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Summary / Samenvatting
Summary

The processing of dead organic matter, also known as detritus, is a central ecosystem process driven by detritus feeding organisms that are mostly located at the bottom of water bodies where dead organic matter (OM) accumulates. Detritivorous organisms form communities composed of invertebrates, fungi and bacteria that interact with each other and their substrate. Although it is likely that links between benthic biodiversity and OM processing are driven by similar mechanisms across different ecosystem types (forest floors, stream beds, coral reefs), it remains a challenge to identify general drivers of decomposition in benthic detrital food webs in different waters. The aim of this thesis was therefore to unravel interactions between the (functional) composition of invertebrate and bacterial communities, organic matter processing and abiotic variables in two contrasting benthic detrital food webs: one on soft bottom sediments and one on solid substrate, mangrove ecosystems. To this purpose, the following objectives have been set: 1) To evaluate the impact of OM composition on invertebrate-substrate interactions and organic matter processing; 2) To assess the impact of abiotic stressors on invertebrate-substrate interactions and organic matter processing; 3) To quantify the effect of functional diversity of bacteria and invertebrates on organic matter processing.

Part 1: Invertebrate-substrate interactions in soft bottom sediments

Invertebrates in soft bottom sediments differ widely in how they move on top or within the sediment, thereby affecting sediment properties. Chapter 2 evaluates whether bioturbation activities of invertebrates have trait specific influences on detritus processing, bacterial activity and community structure, and redox conditions. It was shown that invertebrates enhance bacterial activity and detritus processing, and bacterial community structure was also significantly modified. Bioturbation and spatiotemporal Eh dynamics were brought forward as functional footprints of benthic detrital food webs.

Toxicants potentially decouple these observed links. Chapter 3 therefore aimed to evaluate how toxicants affect invertebrate bioturbation and overall decomposition. It was demonstrated that decomposition can be more sensitive to copper than invertebrates, hinting a decoupling of invertebrate community composition and ecosystem functioning upon stress.

A standard litter surrogate with adjustable chemical composition was deemed necessary for further studies on decomposition. Chapter 4
proposes and tests the use of a decomposition and consumption tablet (DECOTAB) consisting of cellulose powder embedded in an agar matrix to evaluate decomposition and consumption rates in aquatic environments. It was demonstrated that DECOTABs provide a novel, versatile tool to address long-standing questions in aquatic ecology and environmental assessment.

Effects of solar radiation on bacterial communities residing in sediments remain completely unexplored. Chapter 5 therefore investigated the influence of mimicked solar radiation on bacterial functional diversity in laboratory sediments. It was shown that a combined effect of light and OM shapes the functional composition of microbial communities developing in sediments, and acts as an important sorting mechanism for bacterial communities in wetlands.

Different functional metrics of decomposition exist, but are seldom studied in coherence. Chapter 6 tested the response of bacterial functional diversity and activity, biogenic mixing depth and detritus processing to activities of invertebrate species combinations in outdoor mesocosms. By studying several functional parameters in coherence we demonstrated that different ecosystem processes responded differently to invertebrate species composition, and that solar radiation can decouple invertebrate-bacterial interactions.

Part 2: Sponge-environment interactions in mangrove stands

Chapter 7 describes and quantifies the diversity and abundance of mangrove associated sponges of Curaçao and Aruba and correlates variability of regional sponge diversity with environmental variables measured along the surveyed sites. Tannin concentrations vary between mangrove roots, and were correlated to sponge cover as a possible cause for habitat heterogeneity on a smaller scale. It was shown that sponge diversity could be partly explained by the distance towards adjacent reefs and degree of eutrophication. Tannin concentrations did not determine species heterogeneity such as a priori postulated, but were positively related to sponge cover for reasons not yet elucidated.

A positive correlation between sponge coverage and tannin concentrations in prop roots of *Rhizophora mangle* L. was demonstrated in chapter 7, yet the role of tannins within the mangrove sponge association remains uncertain. Chapter 8 tested whether tannins play a role in sponge recruitment and assessed tannin and polyphenol production in *R. mangle* roots in response to sponge colonization. It was demonstrated that tannins are positively involved in larval recruitment of the sponge *Tedania ignis* and that roots significantly enhanced tannin and polyphenolic content in
response to natural and experimental sponge fouling, potentially pointing to a positive feedback in recruitment.

Sponge communities found in Caribbean mangroves are very distinct from sponge communities on nearby reefs, which is potentially caused by bacterial symbionts of sponges capable of degrading recalcitrant substrates. In chapter 9, tannase activity and ability to degrade mangrove-derived DOM was tested in a random set of sponge species. It was demonstrated that sponges commonly associated with mangrove roots contain bacteria that are capable of degrading mangrove-derived DOM, while bacterial communities associated with sponges that are more typical to reef environments appear less proficient in degrading mangrove-derived DOM, potentially leading to the ecological separation between mangrove and reef sponge communities.

A number of sponge species commonly found on reefs are known to deteriorate when transplanted to mangrove roots. Chapter 10 aimed to elucidate the relative importance of substrate and habitat in the ability of sponges to persist in mangrove ecosystems, and to evaluate the role of bacterial symbiont composition and carbon uptake in sponge distribution. It was shown that the inability of typical reef species to survive in mangrove ecosystems is related to habitat and substrate.

It was demonstrated in chapter 10 that the root substrate is of critical importance limiting survival of typical reef species. Chapter 11 therefore evaluates whether dissolved organic matter (DOM) leaching from mangrove roots is responsible for this observation. It is demonstrated that mangrove sponge are not affected by mangrove-DOM, while reef species showed substantial necrosis when exposed to mangrove-DOM, suggesting that mangrove-DOM confines the composition of sponge communities in mangrove ecosystems.

Invertebrate fauna, free living in sediments and fauna attached to solid substrates (sponges) were found to facilitate the activity and composition of bacterial consortia, while vice versa bacterial development conditioned organic matter that was either refractive (peat) or less palatable (mangrove phenolics) for fauna. These refractive components of mangrove organic matter structured the sponge species distribution over mangrove and coral reef substrates. Biotic interactions appeared to be affected via faunal bioturbation modifying sediment redox conditions, or immobilization thereof by a model toxicant (Cu). It was argued that pollutants can affect ecosystem processes before biodiversity is affected. For that reason functional parameters for measuring ecosystem integrity are promoted. Solar radiation influenced the redox conditions of the sediment, thereby masking the concurrent effects of bioturbation, and
overriding the faunal effects on sediment bacteria. Although biodiversity or species composition has served as a touchstone for environmental protection, throughout the present study I found better explanations for detritus decay dynamics when using functional parameters of fauna and bacterial consortia.
Samenvatting

De verwerking van dood organisch materiaal, ook wel bekend als detritus, is een belangrijk ecosysteem proces gedreven door detritivore organismen en die meestal plaatsvindt op de bodem van waterlichamen waar dood organisch materiaal (OM) accumuleert. Detritivore gemeenschappen bestaan uit ongewervelden, schimmels en bacteriën en worden beïnvloed door elkaar en door het substraat. Hoewel het waarschijnlijk is dat connecties tussen biodiversiteit en de verwerking van OM wordt gedreven door soortgelijke mechanismen in verschillende soorten ecosystemen (bosvloeren, stroom bedden, koraalriffen), is het een uitdaging om algemene stuurfactoren van OM-verwerking in contrasterende benthische voedselwebben te identificeren. Het doel van dit proefschrift was dan ook om de interacties te ontrafelen tussen de (functionele) samenstelling van ongewervelde en bacteriële gemeenschappen, de verwerking van OM en abiotische variabelen in twee contrasterende benthische voedselketens: een op zachte zand bodem en een op vast substraat, mangrove-ecosystemen. Hiervoor zijn de volgende doelstellingen vastgesteld: 1) De gevolgen van OM samenstelling op ongewervelde-substraat interacties en verwerking van OM te evalueren; 2) De gevolgen van abiotische stressoren op ongewervelde-substraat interacties en verwerking van OM te evalueren; 3) Het effect van functionele diversiteit van bacteriën en invertebraten op het verwerken van OM te kwantificeren.

Deel 1: Invertebrate-substraat interacties in zachte zand bodems

Ongewervelden die in zachte sediment leven verschillen sterk in de manier waarop ze bewegen op of in het sediment. Hoofdstuk 2 beoordeelt of bioturbatie activiteiten van ongewervelde dieren specifieke invloeden hebben op de verwerking van OM, de bacteriële activiteit en samenstelling, en redox condities. Er werd aangetoond dat ongewervelden de bacteriële activiteit en detritus verwerking verhogen, en de samenstellingen van de bacteriegemeenschappen was significant veranderd. Bioturbatie en tijdruimtelijke Eh dynamiek werd naar voren gebracht als functionele voetsporen van benthische voedselketens.

Toxische stoffen ontkoppelen mogelijk deze geobserveerde koppelingen. Hoofdstuk 3 evalueerde daarom hoe toxische stoffen invloed hebben op bioturbatie door ongewervelden en de verwerking van OM. Er werd aangetoond dat OM afbraak gevoeliger kan zijn voor koper dan ongewervelde, waardoor een ontkoppeling van de ongewervelde gemeenschap en het functioneren van ecosystemen door stress zichtbaar was.
Een standaard test substraat met een aan te passen chemische samenstelling is noodzakelijk voor verdere studies over OM afbraak. Hoofdstuk 4 test daarom het gebruik van een afbraak en consumptie tablet (DECOTAB) bestaande uit cellulosepoeder ingebed in een agar matrix die het mogelijk maakt de afbraak en consumptie te evalueren in aquatische milieus. Er werd aangetoond dat DECOTABs een veelzijdig instrument is om langstaande vragen in aquatische ecologie en milieu-beoordeling te beantwoorden.

Effecten van zonnestraling op benthische bacteriële gemeenschappen is volledig onontgonnen. Hoofdstuk 5 onderzocht daarom de invloed van nagebootste zonnestraling op de bacteriële functionele diversiteit in laboratorium sedimenten. Er werd aangetoond dat een gecombineerd effect van licht en OM belangrijk is voor de ontwikkeling van de functionele samenstelling van bacteriële gemeenschappen.

Verschillende functionele parameteres van ontbinding bestaan, maar worden zelden bestudeerd in samenhang. Hoofdstuk 6 evalueerde bacteriële functionele diversiteit en activiteit, biogene mengdiepte en OM afbraak in relatie tot de activiteiten van verschillende combinaties van ongewervelden in mesocosms. Door het bestuderen van verschillende functionele parameters werd aangetoond dat verschillende ecosysteem processen verschillend reageren op soortensamenstelling, waarin zonnestraling de interacties tussen ongewervelde-bacteriële kan ontkoppelen.

Deel 2: Spons-milieu-interacties in mangrove systemen

Hoofdstuk 7 beschrijft en kwantificeert de diversiteit en rijkdom van mangrove-geassocieerde sponzen van Curaçao en Aruba en correleert variabiliteit van diversiteit met omgevingsvariabelen gemeten langs de onderzochte sites. Tannine concentraties variëren tussen de mangrove wortels, en werden gecorreleerd aan sponsbedekking als een mogelijke oorzaak voor heterogeniteit op een kleinere schaal. Er werd aangetoond dat spons diversiteit gedeeltelijk kan worden verklaard door de afstand naar aangrenzende riffen en de mate van eutrofiëring. Tannine concentraties waren positief gerelateerd aan sponsbedekking.

Een positieve correlatie tussen sponsbedekking en tannine concentraties in wortels van *Rhizophora mangle* L. werd aangetoond in hoofdstuk 7, maar de rol van de tannines in de mangrove-spons associatie blijft onzeker. Hoofdstuk 8 test of tannines een rol spelen bij spons werving en beoordeeld of tannine en polyfenol productie in *R. mangle* wortels een reactie is op spons kolonisatie. Er werd aangetoond dat tannines positief betrokken zijn bij werving van larven van de spons *Tedania ignis* en de wortels verhoogde aanzienlijk tannine en polyphenolic.
gehaltes in reactie op natuurlijke en experimentele spons-bedekking, wat mogelijk wijst op een positieve feedback in de werving van larven.

Sponsgemeenschappen in Caribische mangrovenbossen zijn zeer verschillend van sponsgemeenschappen op nabijgelegen riffen. Dit wordt mogelijk wordt veroorzaakt door bacteriële symbionten van sponzen die recalcitrante substraten kunnen afbreken. In hoofdstuk 9 werd tannase activiteit en de mogelijkheid om mangrove-OM af te breken getest in een willekeurige reeks van sponssoorten. Er werd aangetoond dat sponzen die vaak geassocieerd zijn met mangrove wortels bacteriën bevatten die in staat zijn mangrove-OM af te breken, terwijl de bacteriële gemeenschappen in sponzen die meer typisch zijn voor rif omgevingen minder bedreven zijn in het afbreken van mangrove-OM. Dit verklaart mogelijk de ecologische scheiding tussen de mangrove en rif spons gemeenschappen.

Het is bekend dat een aantal sponssoorten algemeen aangetroffen op riffen verslechteren wanneer ze getransplanteerd worden naar mangrove wortels. Hoofdstuk 10 is erop gericht om het relatieve belang van de wortel en habitat te onderzoeken in het vermogen van de sponzen te volharden in mangrove-ecosystemen. De rol van bacteriële gemeenschappen en de opname van koolstof in sponsen werd geëvalueerd. Er werd aangetoond dat het onvermogen van typische rifsoorten om te overleven in mangroven was gerelateerd aan zowel habitat en de wortel.

Er werd aangetoond in hoofdstuk 10 dat de wortel van cruciaal belang is in de beperkte overleving van typische rifsoorten. Hoofdstuk 11 evalueert daarom de vraag of opgelost organische materiaal (DOM) dat lekt uit mangrovewortels hiervoor verantwoordelijk is. Er is aangetoond dat de mangrove-spons niet wordt beïnvloed door mangrove-DOM, terwijl rifsoorten aanzienlijke necrose veroorde bij blootstelling aan mangrove-DOM, wat suggereert dat mangrove-DOM de samenstelling van de spons gemeenschappen in mangrove-ecosystemen beperkt.

Ongewervelde fauna, ofwel vrij levend in sedimenten of gehecht aan vaste substraten (sponzen), hebben een sterke invloed op de de activiteit en de samenstelling van bacteriële gemeenschappen, terwijl omgekeerd bacteriële ontwikkeling afhankelijk was van recalcitrant organisch materiaal (turf en mangrove fenolen). Deze recalcitrante componenten van mangrove organische materiaal beïnvloed de sponsverspreiding. Biotische interacties worden beïnvloed via bioturbatie die redoxcondities in het sediment modificeren, of immobilisatie daarvan door een giftige stof (Cu). Er werd aangevoerd dat verontreinigende stoffen ecosysteem processen kunnen beïnvloeden voordat de biodiversiteit wordt aangetast. Om die reden wordt voorgesteld functionele parameters te gebruiken voor
het meten van de integriteit van ecosystemen. Zonnestraling beinvloedt de redox condities van het sediment, en maskeert de gelijktijdige effecten van bioturbatie. Hoewel biodiversiteit of soortensamenstelling tot op heden waardevol is geweest in de beoordeling van het milieu, suggereert deze studie dat detritus afbraak beter verklaard wordt met het gebruik van functionele parameters van fauna en bacteriële consortia.
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Publications


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