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REGULAR ARTICLE

Bromeliad tanks are unique habitats for microbial communities involved in methane turnover

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Abstract
Background and aims Tank bromeliads collect organic matter and rainwater (= tank slurry) between their densely arranged leaf axils for their nutrient demand. Diverse communities of microorganisms inhabit these tanks and are responsible for the breakdown of organic matter. Anaerobic degradation results in the release of substantial amounts of methane. We hypothesized that each individual bromeliad harbors its own microbial community, which is affected by chemical tank-slurry properties. We further hypothesized that methanotrophic bacteria inhabit bromeliad tank slurries, potentially able to oxidize the produced CH_4.
Methods We investigated communities of Bacteria, Archaea, methanogenic and methanotrophic microorganisms measuring their abundance (qPCR) and composition (TRFLP) within eight bromeliad tanks of the species *Werauhia gladioliflora* sampled in a Costa Rican lowland forest. Tank slurries were analyzed for pH, carbon, nitrogen, oxygen and fatty acid concentrations. Methane oxidation rates were determined in five bromeliad tank slurries.
Results Our results showed that microbial communities differed between plants and were affected by chemical tank slurry properties. Further, not only methanogenic archaea but also methanotrophic bacteria were detected in the tanks of all bromeliad plants, the latter being potentially able to aerobically oxidize between 25 and 62 μg CH_4 gdw^{-1} h^{-1}.
Conclusion Our results indicate that every bromeliad tank is a unique island with respect to its resident microbial community. The presence of methanogens and active methanotrophs in all tank slurries further indicates the potential for both methane formation and methane oxidation.

Keywords Bromeliad · Bacteria · Archaea · Methanogens · Methanotrophs · Methane oxidation

Introduction

The Bromeliaceae represent one of the largest neotropical families of flowering plants containing about 58 genera with ca. 3140 species (Givnish et al. 2011; Balke et al. 2008). The plants grow terrestrially and epiphytically throughout neotropical forest ecosystems. Their densely arranged leaves form tanks that efficiently collect wind-borne particles, leaf litter and rainwater (= tank slurry) for their nutrient demand, and highly increase the amount of stored carbon and water in the canopies of neotropical forests (Nadkarni 1994). Diverse communities of macro- and microorganisms inhabit these tanks and are responsible for the

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breakdown of tank organic matter, the release of plant-available nutrients (Ngai and Srivastava 2006) and the emission of substantial amounts of CH_4 (Martinson et al. 2010) and CO_2 (Atwood et al. 2013). Tank bromeliads were used as natural model systems to study food web structures (Kitching 2001; Srivastava 2006; Srivastava et al. 2008; Brouard et al. 2011), animal richness (Richardson 1999), activity and distribution of (aquatic) invertebrates (Carrias et al. 2001; Marino et al. 2013) and microorganisms (Carmo et al. 2014). Many different bacteria and archaea, which are commonly found in soils, were also detected in Ecuadorian and Costa Rican tank bromeliad slurries (Goffredi et al. 2011a, 2011b; Martinson et al. 2010). Due to their high abundance tank bromeliads may therefore represent important habitats for microorganisms involved in the cycling of carbon and nitrogen (Goffredi et al. 2011b). Recently, Carmo et al. (2014) observed a large variation in the community structure when investigating the bacteria in the tank water of different *Neoregelia cruenta* bromeliads. Similar results were reported by Farjalla et al. (2012) evaluating the microbial community in the tank water of four bromeliad species. They showed that bacteria had no habitat association and thus concluded stochastic colonization processes. Environmental controls on microbial community composition in bromeliad tanks have hardly been investigated. Previous studies have shown that exposure to sun or shade affects the composition and diversity of aquatic insect in the water of bromeliads (Lopez and Rios 2001). However, Carmo et al. (2014) reported that bacterial communities in tank water showed no grouping by the environmental factors tested (e.g season, sun exposure). The bacterial communities in Costa Rican tank bromeliads were affected by acid-base conditions (Goffredi et al. 2011b). However, it should be noted that sampling strategies were different in previous studies using either the tank water (Carmo et al. 2014; Farjalla et al. 2012) or tank slurry to investigate bacterial communities (Goffredi et al. 2011b). These differences complicate a direct comparison between the studies and may possibly lead to an underestimation or exclusion of resident microbial organisms.

This is the first study investigating the bacterial and archaeal microbial communities as well as the functional groups of methanogens and methanotrophs in the tank slurry of individuals of the species *Werauhia gladioliflora*. We tested the following hypothesis: (1) each bromeliad plant represents a unique island with respect to its microbial community driven by chemical tank-slurry properties. We further investigated the variability of microbial community composition and abundance within single bromeliads. Since tank bromeliads contribute to the methane cycle in neotropical forests (Martinson et al. 2010) we further hypothesized (2) that methanotrophic bacteria inhabit bromeliad tank slurries, potentially able to oxidize the produced CH_4.

In order to reduce potential variability of microbial communities, we tested our hypotheses by selecting eight mature tank bromeliads of the species *Werauhia gladioliflora*, which were of similar size and were grown epiphytically in the same habitat within one hectare of a Costa Rican secondary lowland forest. The abundance and composition of microbial groups were determined targeting the bacterial and archaeal 16S rRNA gene as well as the methanogenic and methanotrophic marker genes *mcrA* and *pmoA*, respectively, using quantitative PCR (qPCR) and terminal restriction fragment length polyphormism (TRFLP). We measured the main chemical tank-slurry properties for each individual plant (pH, oxygen, carbon, nitrogen and fatty acid concentrations) and tested their explanatory power for microbial communities. The potential of aerobic methane oxidation was further determined in five tank slurries using incubation experiments.

Material and methods

Sampling site and chemical analysis

The field station La Gamba, situated in the southwest of Costa Rica on the edge of the National Park Piedras Blancas (N 8°42′61″, W 83°12′97″) encircles an area of 142 km^2 and is one of the last pristine rainforests in the lowlands at the Pacific coast in Central America. The climate in this region is dominated by a high rainfall (6100 mm y^{-1}; Hofhansl et al. 2014). The area around the field station is dominated by tank bromeliads of the genus *Werauhia* (syn. *Vriesea*).

For this study, we collected eight accessible and epiphytically grown tank bromeliads (A-H) of the species *Werauhia gladioliflora* at breast height in an area of approximately 1 ha next to the field station in May 2012 during the wet season. Since we focused on microorganisms which are involved in the methane cycle we sampled during the wet season as water-filled bromeliads enhance the creation of anoxic niches and therefore

methane formation. The plant height and diameter ranged between 82–94 cm and 130–140 cm, respectively. Tank bromeliads were carefully detached from their host trees avoiding loss of water or debris. The tank slurry is defined here as the impoundments of rainwater-soaked organic debris located between tank bromeliad leaf axils. For comparison between plants, tank slurries from three different leaf axils were sampled from each of the eight different plants (e.g. A1, A2, A3). For comparison within plants, four out of the eight bromeliads were chosen and three additional leaf axils at opposite position were sampled. For molecular analysis we took sample volumes of 2 g in 2-ml Eppendorf microcentrifuge tubes and for incubation experiments 5–10 g in 50-ml Falcon tubes for five out of the eight individuals. Additionally, three soil samples were taken from the study site and used for TRFLP analysis.

Tank slurry and soil samples were used for chemical and molecular characterization

Dissolved oxygen concentration in the slurry of the central tank was directly measured in the field by a GMH 3630 Digital Oxymeter (Greisinger). The pH of each tank slurry was measured using a pocket-sized pH meter (HI98103 Checker®, Hanna Instruments). Tank slurries were immediately transported in cooled state (4 °C) to the Max-Planck-Institute for Terrestrial Microbiology in Marburg, Germany and then stored frozen at −20 °C until analysis. The supernatant of centrifuged tank slurry was filtered through cellulose membranes (0.2 μm) and analyzed for fatty acids and ion concentrations using high performance liquid chromatography (HPLC; Sykam) with refractive index and UV detectors and ion chromatography (Sykam), respectively (Krumböck and Conrad 1991; Bak et al. 1991). Fatty acid and ion concentration was calculated using dilution series of external standards (Sigma-Aldrich, Carl Roth). Total carbon and nitrogen concentration of tank slurries and soil samples were analyzed on a CHNS-elemental analyzer (Analytical Chemical Laboratory, University of Marburg).

Molecular analyses

DNA was extracted from 0.3 g (fresh weight) tank slurry using the NucleoSpin® Soil Kit (Machery-Nagel) according to the manufacturer's protocol. The DNA was dissolved in 50 μL nuclease-free water and checked for quality and quantity using a ND1000 spectrophotometer (NanoDrop). DNA was used for all subsequent molecular analyses (qPCR, TRFLP).

Polymerase chain reactions (PCR) for TRFLP analysis targeted bacterial and archaeal 16S rRNA genes as well as the methanogenic marker gene *mcrA,* coding for the methyl coenzyme M reductase subunit A*,* and the methanotrophic marker gene *pmoA*, coding for the methane monooxygenase subunit A. Primer combinations used in the study were: 27f/907r for Bacteria (Osborne et al. 2005; Muyzer et al. 1995) with FAM-labeled forward primer; 109f/934r for Archaea (Großkopf et al. 1998) with FAM-labeled reverse primer; MCRf/MCRr (Springer et al. 1995) for methanogens with FAM- labeled forward primer; A189f/mb661r (Costello and Lidstrom 1999) for methanotrophs with FAM-labeled forward primer. PCR was carried out in a total volume of 50 μL, containing 200 μM desoxynucleotriphosphates (Fermentas), 1× GoFlexiGreen Buffer (Sigma- Aldrich), 10 μg Bovine Serum Albumin (BSA; Roche), 1 U GoTaq DNA Polymerase (Sigma-Aldrich), 0.5 μM of each primer pair, 1.5 mM $MgCl_2$ (Promega) and 20 ng template DNA. The PCR reaction was carried out for 94 °C for 3 min followed by 22 cycles for bacterial and 30 cycles for archaeal, *mcrA* and *pmoA* gene amplification of 94 °C for 30 s, 52 °C for 45 s, 72 °C for 90 s and a single step of final elongation of 72 °C for 5 min. PCR products were analyzed by 1.5 % agarose gelelectrophoresis and visualized by staining with Gel Red™ (Biotium) for 30 min. Bands were cut out and purified using Wizard® SV Gel and PCR Clean-Up System (Promega) according to the manufacturer's protocol. Afterwards 100 ng of purified PCR product was digested in a total volume of 10 μL with 2.5 Units of restriction enzyme and the provided reaction buffer with a 1× final concentration. The archaeal 16S rRNA and the *pmoA* amplicons were digested for 6 h at 36 °C using *TaqI* enzyme (Promega), 16S bacterial amplicons were digested at 37 °C for 15 min using the *MSPI* Fast Digest® restriction enzyme (Fermentas). The *mcrA* amplicons were digested with *Sau96I* FastDigest® (Fermentas) for 15 min at 36 °C. All samples were purified using the SigmaSpin Post-Reaction Clean-up Columns (Sigma-Aldrich) according to the manufacture's protocol. The size separation was conducted on an ABI PRISM 3130 capillary Genetic Analyzer (Applera) using the software Genescan 4.0 (Applied Biosystems). The TRFLP data were obtained by

comparison with an internal DNA standard (X-rhodamine MapMarker® 1000, BioVentures,

Murfreesboro). The resulting TRFLP profiles were standardized as described in Dunbar et al. (2001) using integrated peak area. Standardization often eliminates peaks from profiles with a large total peak area by reducing some peak area below the threshold. In brief, the sample with the smallest DNA quantity (total fluorescence) and the sample with the largest DNA quantity were used to determine a correction factor (the quotient of total fluorescence of the sample with the smallest DNA quantity / total fluorescence of the sample with a larger DNA quantity). This factor was then multiplied with each peak of the DNA profiles with high total fluorescence. Peaks which were then below the 1 % threshold were deleted. The absolute numbers of bacterial and archaeal 16S rRNA, *mcrA* and *pmoA* genes were determined by quantitative PCR (qPCR) using the primer combination Ba519f/Ba907r (Stubner 2002) for bacteria, Ar364f/Ar934br (Burggraf et al. 1997/ Großkopf et al. 1998) for archaea, MCRf/MCRr (Springer et al. 1995) for methanogens and A189F/ mb661 (Costello and Lidstrom 1999) for methanotrophs. The qPCR was set up in 96-well microtiter plates (BioRad) using a SYBR green I assay. Each qPCR reaction contained in a total volume of 25 μL 1× SYBR®Green Ready Mix™ (Sigma), 3 mM $MgCl_2$ (Sigma), 0.66 μM of each primer and 1 μM FITC (fluorescein isothiocyanat, BioRad) as well as 2 μL of template. Technical replicates were done using 1:10 and 1:100 dilutions of the template DNA. Negative controls without matrix DNA were run in parallel to ensure purity of the used reagents. For all qPCR assays, standards containing known numbers of DNA copies of the target gene were used. The quantification standard was applied in a dilution series with 10^1–10^7 gene copies. Thermoprofiles for the quantification of the archaeal 16S rRNA gene copies and *mcrA* gene copies are described by Angel et al. 2011. The quantification of the bacterial 16S rRNA gene was done using the following thermoprofile: initial denaturation 94 °C for 8 min, then 45 cycles of 94 °C for 20 s, 50 °C for 20 s and 72 °C for 50 s. The quantification of *pmoA* genes was performed with the following thermoprofile:initial denaturation step of 94 °C for 6 min, then 50 cycles of 94 °C for 25 s, 65.5 °C for 20 s and 72 °C for 35 s for annealing and signal reading. After each quantification a melting curve was performed to ensure the purity of the PCR products.

Methane oxidation

Considering that microbial communities showed no differences within individual plants we used tank slurries of various leaf axils from one plant. Five individual tank bromeliads (A, B, D, F, H) were used to determine aerobic methane oxidation rates. Tank slurries (5–10 g) were incubated in sterile 120 ml serum bottles closed with gas-tight gray butyl stoppers at 25 °C under aerobic conditions with 1 % CH_4 (Messer) in the headspace. Tank slurries were wet (gravimetric water content 87 ± 2 %) but not covered with water. Gas samples were taken immediately after the addition of CH_4 and then after 8, 24 and 32 h of incubation using a gas-tight pressure lock syringe (Vici) and analyzed using a gas chromatograph with a flame ionization detector (Shimadzu). Methane oxidation rates (μg CH_4 gdw^{-1} h^{-1}) were calculated between 0 and 24 h of incubation for individual A, B, F, H and between 8 and 32 h for individual D using linear regression.

Statistical analysis

All statistical analyses were conducted in R version 2.10.1 (R Core Team 2013). Data of qPCR and chemical properties were tested for normality by Kolmogorov-Smirnov test and for homogeneity of variance by Levene's test. If necessary, normal distribution was achieved by log-transformation of the data. Means were compared using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Non-metric-multidimensional scaling analysis (NMDS) of microbial community composition was performed based on TRFLP data using the Bray-Curtis dissimilarity index. For NMDS analysis we reported a stress value (in %) indicating the lack of the fit between the dimensional mapping of the dissimilarities and the original dissimilarities. Differences between community compositions within a tank bromeliad and among tank bromeliads were investigated using *permutational* multivariate analysis of variance (Permanova) based on TRFLP results. Microbial community structure was related to tank slurry properties using canonical correspondence analyses (CCA). Variables which were highly different between tank bromeliads (carbon, nitrogen, oxygen concentration and pH; $P < 0.001$; Table 1) were included in the CCA model for which significance was tested using ANOVA. The relative contribution

Table 1 Differences in environmental variables among the eight tank bromeliad slurries of *W. gladioliflora* were determined by a one-way analysis of variance (ANOVA)

Variable	df	F	*P*-value
pH	7	14.0	<0.001*
C_{tot} (%)	7	14.5	<0.001*
N_{tot} (%)	7	14.6	<0.001*
Oxygen (%)	7	17.0	<0.001*
Acetate (mM)	7	2.3	0.08
Lactate (mM)	7	1.4	0.29

*= significant

of each variable to the total constrained variation was calculated by variation partitioning according to Borcard et al. (1992) and Legendre (2008). Linear regression was used to test for a relationship between copy numbers of the bacterial 16S rRNA, archaeal 16S rRNA, *mcrA* and *pmoA* gene to tank slurry properties carbon, nitrogen, oxygen, pH). ANOVA was done using the *stats* package. NMDS, Permanova and CCA analyses were done using package *vegan*. All levels of significance were defined at $P < 0.05$.

Results

Tank slurry of *Werauhia gladioliflora*

The tank slurries showed total carbon and nitrogen concentrations of 39.8 ± 6.9 % and 1.7 ± 0.3 % (Supplementary Table S1), respectively, which differed between individual plants (Table 1). By comparison, the total carbon content of the adjacent soil was eight times lower (4.9 ± 1.8 %) and the total nitrogen concentration six times lower (0.3 ± 0.1 %). The pH of tank slurry and the oxygen concentration of tank water ranged between pH 4.6–6.2 and 2.9–9.6 mg O_2/L, respectively, and differed between individual tank bromeliads (Table 1). Concentrations of lactate, acetate and formate ranged between 0.01–2.93 mM, 0.02–0.47 mM and 0.05–1.15 mM, respectively (Supplementary Table S1). Concentrations of fatty acids did not differ significantly between the individual tank bromeliads except formate (Table 1). Nitrate was not detectable in any sample.

Microbial communities in *Werauhia gladioliflora*

Bacterial 16S rRNA gene copies ranged from 3.7×10^{10} to 1.8×10^{11} copies gdw^{-1} (Fig. 1). Archaeal 16S rRNA gene copy numbers were one order of magnitude lower than bacterial gene copy numbers. The functional marker gene *mcrA* showed more than 10^9 copies in seven bromeliads (Fig. 1). The *pmoA* marker gene was determined in five individual tank slurries with more than 10^8 copies (Fig. 1). Significantly lower *pmoA* gene copies ($< 5 \times 10^6$ gene copies gdw^{-1}) were detected in tank bromeliad individuals E and G. The latter one showed significantly lower archaeal 16S rRNA gene and *mcrA* gene copies as well (Fig. 1). Within individual plants no significant differences were observed between copy numbers of bacterial and archaeal 16S rRNA as well as *mcrA* genes (Supplementary Table S2). Solely, the *pmoA* gene copy numbers within individual D were significantly different (Supplementary Table S2).

Linear regression was used to test the relationship between copy numbers of the bacterial 16S rRNA, archaeal 16S rRNA, *mcrA* and *pmoA* gene and tank slurry properties (carbon, nitrogen, oxygen, pH). Solely the number of *pmoA* marker genes correlated significantly with the oxygen concentration in the bromeliad tanks. With increasing oxygen concentration the abundance of the methanotrophic community apparently increased in tank slurries of *W. gladioliflora* (Fig. 2a).

Within individual tank bromeliads no differences were detected between microbial community compositions targeting the bacterial 16S rRNA gene, except for individual C (Supplementary Table S2). Among tank bromeliads bacterial, archaeal as well as methanogenic and methanotrophic community composition differed. This is demonstrated by the *P*-values of the Permanova analysis which were significant for the archaeal ($P = 0.004$) and methanotrophic ($P = 0.002$) community and highly significant for the bacterial and methanogenic community ($P = 0.0009$). The differences of the *P*-values were also indicated in NMDS-plots. The bacterial and methanogenic communities displayed a more distinct clustering between individual plants (Fig. 3 a, c) than the archaeal or methanotrophic communities (Fig. 3 b, d). The archaeal and methanotrophic communities were represented by TRFs which were found in similar abundances in most of the tank bromeliads (Supplementary Fig. S1). For instance, the archaeal community was throughout dominated by a 393-bp TRF. In 7 bromeliads the methanotrophic community

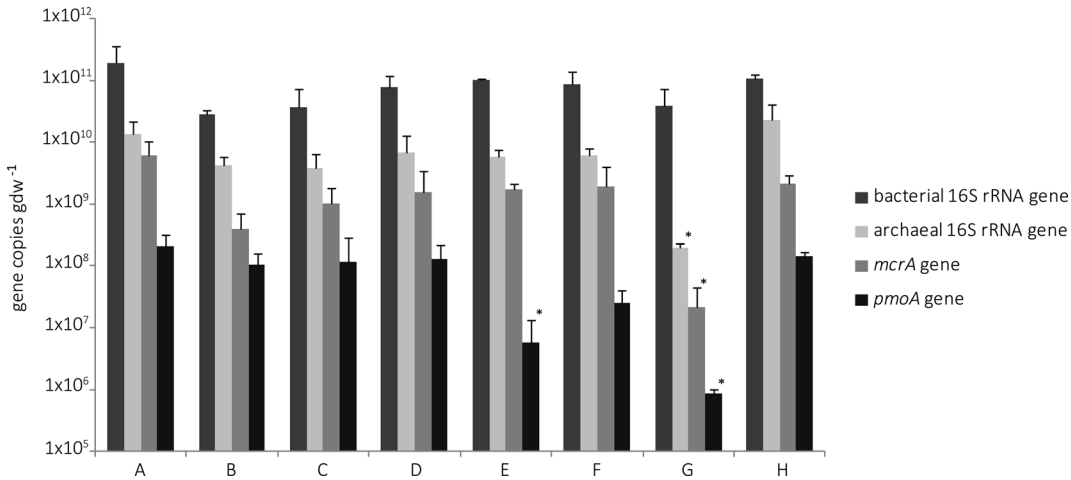

Fig. 1 Gene copy numbers of bacterial, archaeal, methanogenic and methanotropic communities in the slurry of eight individual tank bromeliads of the species *W. gladioliflora* targeting the bacterial and archaeal 16S rRNA gene and the methanogenic and methanotrophic marker gene *mcrA* and *pmoA*, respectively. Differences in the copy numbers between individual plants (A-H) were determined by a one-way analysis of variance (ANOVA) followed by a Tukey's post hoc test and indicated by an asterisk (*). Error bars represent standard deviation ($n = 3$)

was dominated by a 245-bp TRF representing more than 50 % of the relative abundance. Based on NMDS plots representing the archaeal community individual B may be proposed as an outlier (Fig. 3 b). Nevertheless, even after the exclusion of the TRFLP data of individual B for Permanova analysis the archaeal community composition between the remaining tank bromeliads differed. The NMDS plot of the methanotrophic community may represent individual G as an outlier (Fig. 3 d). After removal of TRFLP data of individual G from the Permanova analysis the methanotrophic community composition between the remaining tank bromeliads did not differ anymore. Canonical correspondence analyses (CCA) based on TRFLP community profiles were carried out to evaluate the effects of tank slurry properties on microbial communities in tank bromeliads. Carbon, nitrogen and oxygen concentrations together with pH affected the bacterial, archaeal, methanogenic and methanotrophic community in tank bromeliads explaining 27 %, 28 %, 32 % and 30 % of their variability, respectively (Fig. 4 a-d). Variations in community compositions were significantly explained by the first CCA axis ($P \leq 0.01$). The first axis sorted the bacterial, archaeal and methanogenic community composition along a gradient from high C and N and low pH and oxygen concentrations to tank substrates with low C

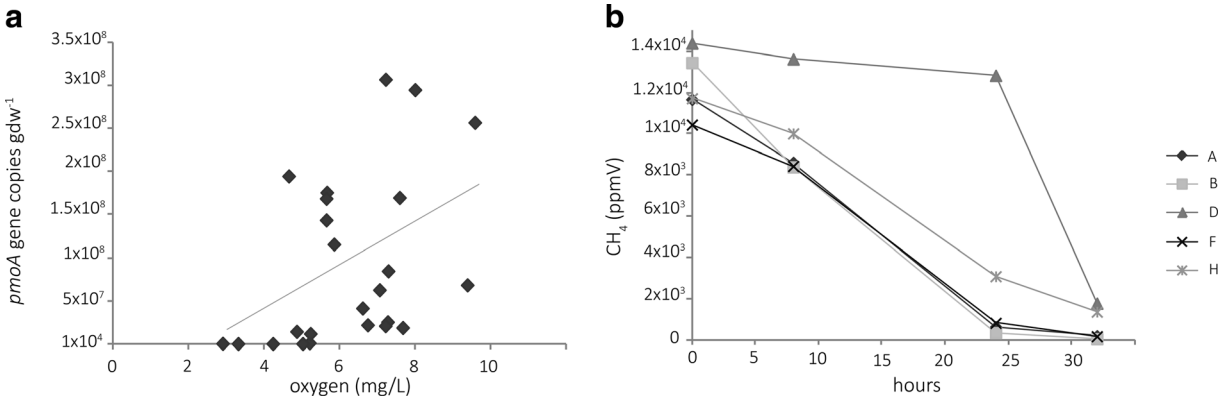

Fig. 2 **a** Linear regression showing the relationship between oxygen concentration and *pmoA* gene copy numbers in tank slurries of *W. gladioliflora* (*pmoA* copies = $-6.5 \times 10^7 + 2.5 \times 10^7 \times$ oxygen concentration; adj. $r^2 = 0.15$; $p = 0.03$; $n = 24$). **b** Temporal change of CH_4 concentration in the headspace of flasks with tank slurry sampled from five plants of the species *W. gladioliflora* (A, B, D, F, H).

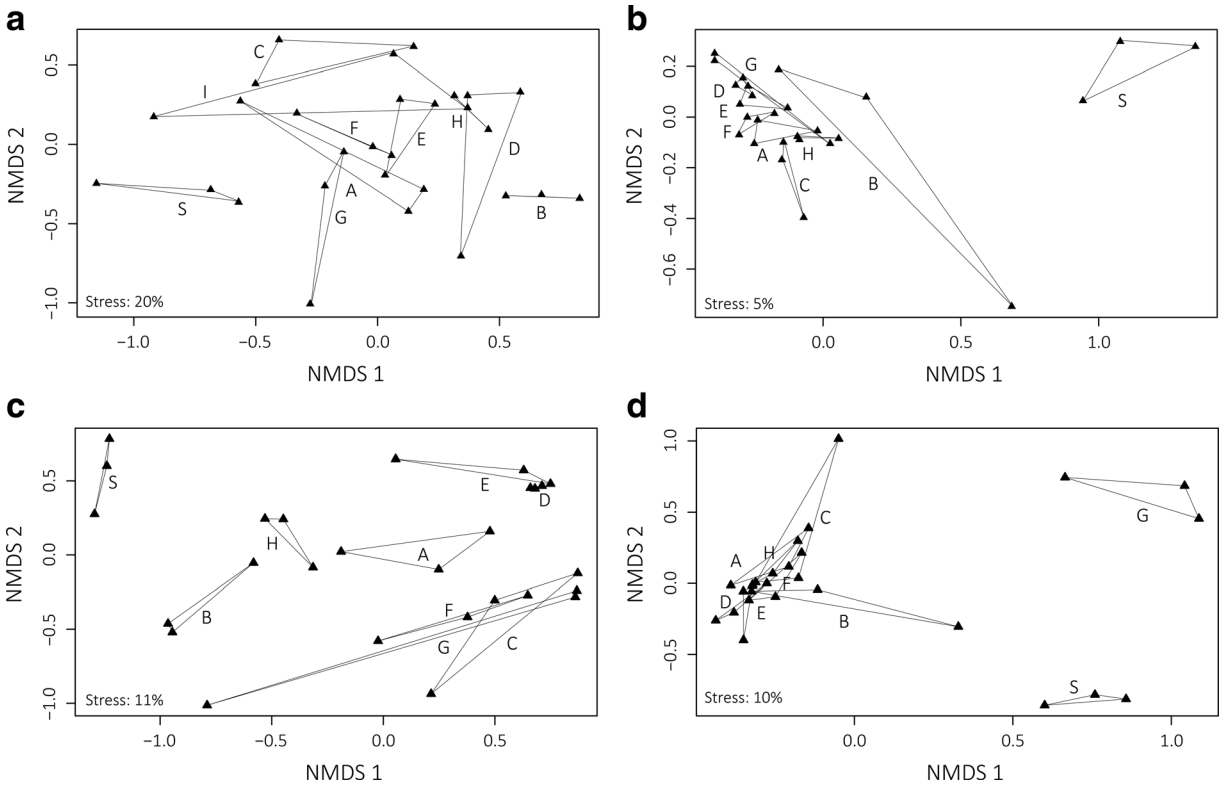

Fig. 3 Non-metric-multidimensional scaling analysis of the microbial community composition in bromeliad tank substrates of *W. gladioliflora* based on Bray–Curtis dissimilarities using TRFLP profiles of the bacterial 16S rRNA gene (**a**), archaeal 16S rRNA gene (**b**), methanogenic marker gene *mcrA* (**c**) and methanotrophic marker gene *pmoA* (**d**). Three replicates per plant (A-H) are connected to a triangle and represent the community in a single tank bromeliad and the respective microbial community in nearby sampled soil (S). The closer two points are the more similar they are in community composition. The stress values (in %) indicate the lack of the fit between the dimensional mapping of the dissimilarities and the original dissimilarities

and N, and high pH and oxygen concentrations. Carbon concentration was a fundamental factor controlling the bacterial, archaeal and methanogenic community composition (Table 2). For instance, more than 20 % of the archaeal community variation was explained by carbon concentration in tank slurry. The methanotrophic community was mainly sorted by oxygen concentration (Fig. 4 d) which alone explained more than 13 % of the variation (Table 2).

Aerobic methane oxidation in *Werauhia gladioliflora*

Considering the observation that the slurries were well mixed within each bromeliad we used pooled slurry from different leaf axils of one plant to investigate the potential of methane oxidation. Aerobic methane oxidation was investigated in tank slurries from five plants during incubation experiments. Methane concentration (ppm) in the headspace of all incubation flasks decreased with time (Fig. 2b). The oxidation rates ranged between 25 and 62 μg CH_4 gdw^{-1} h^{-1} (Table 3).

Discussion

In this study, copy numbers of the methanogenic marker gene *mcrA* in Costa Rican tank bromeliads were as high or even higher than those in a flooded rice field soil (Watanabe et al. 2009; Ma et al. 2012), the latter one representing a major source of atmospheric methane (Conrad 2002). Interestingly, methane oxidizing bacteria, quantified by the methanotrophic marker gene *pmoA,* were also found in all eight tank slurries in numbers that were in the same range as observed in wetland sediments or rice field soils (DeJournett et al. 2007; Wu et al. 2009). Aerobic methanotrophic bacteria are a highly specialized group of microbes using methane as sole source of energy and carbon (Hanson and

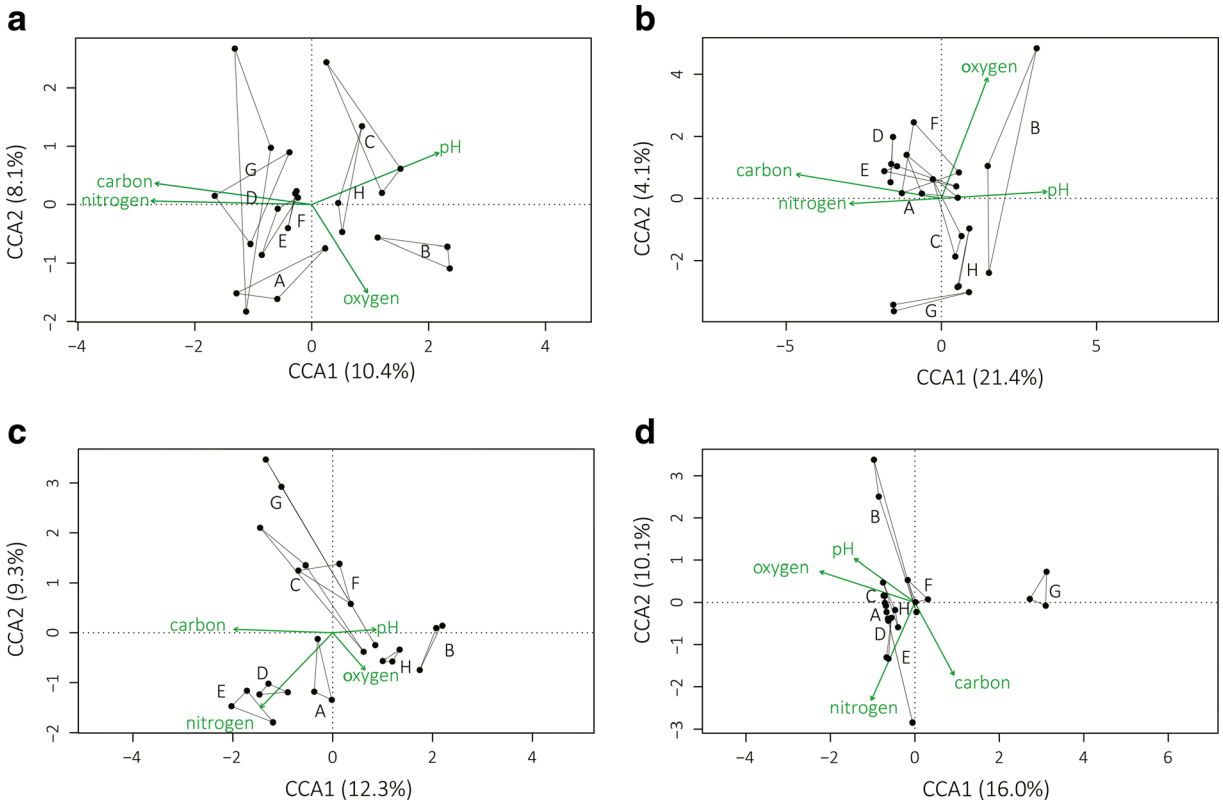

Fig. 4 Ordination of the bacterial (**a**), archaeal (**b**), methanogenic (**c**) and methanotrophic (**d**) community across the axis of a canonical correspondence analysis based on TRFLP community profiles. Three replicates per plant (A-H) are connected to a triangle and represent the community in a single tank bromeliad of the species *W. gladioliflora*. The shared effect of carbon, nitrogen, oxygen concentration and pH of tank slurry on the respective community was for each model significant

Hanson 1996; Murrell and Jetten 2009) and therefore potentially mitigate methane emission (Frenzel et al. 1990). The abundance of the *pmoA* gene was positively correlated with the concentration of oxygen which serves as electron acceptor in methane oxidation and together with methane concentration limits methane oxidation activity. In summary, the presence of methanogens and methanotrophs in all eight individual bromeliads indicates the operation of both methane formation and methane oxidation in bromeliad tanks. We cannot exclude potential anaerobic CH_4 oxidation processes but the operation of aerobic CH_4 oxidation was confirmed by the decrease of CH_4 concentration during the incubation of tank slurry under aerobic conditions. Our results support the hypothesis that methanotrophic bacteria are active in tank bromeliads especially when tank slurry is exposed to oxygen. Tank bromeliads are in general very efficient in maintaining water in their tanks and therefore may create anoxic niches where methanogenesis can occur. However, tank slurries can be regularly exposed to oxygen and during periods of droughts tank slurries can completely dry out (Zotz and Thomas 1999). With the addition of 1 % CH_4 we solely targeted low-affinity methanotrophs that are adapted to grow at high CH_4 concentrations (Dunfield 2007). Along with dry periods methane emission decreases in tank slurries (Brandt et al. 2014) that may also favor the activity of high-affinity methanotrophs in bromeliad tanks.

Epiphytic bromeliads increase the volume of arboreal carbon storage by creating catchments in which litter accumulates until it is decomposed (Nadkarni 1994). Pittl et al. (2010) detected higher bacterial cell numbers in this decomposed litter within tank bromeliads ($\geq 10^{10}$ copies gdw^{-1}) than in tropical canopy soils (taken from the humus pockets generated around tank bromeliad roots) or terrestrial soil, the latter showing the lowest abundance. In this study, bacterial copy numbers in tank bromeliad slurry were found to be up to one order of magnitude higher ($\leq 1.8 \times 10^{11}$ copies gdw^{-1}) than in

Table 2 Relative contribution of each environmental variable to the total constrained variation for the bacterial, archaeal, methanogenic and methanotrophic microbial community composition in bromeliad tank slurry of *W. gladioliflora*

Community	Variable	% Explained	*P*-value
Bacteria	Carbon	10.0	0.005*
	Nitrogen	7.3	0.005*
	pH	8.4	0.01*
	Oxygen	6.6	0.03*
Archaea	Carbon	20.8	0.005*
	Nitrogen	9.7	0.06
	pH	11.8	0.03*
	Oxygen	5.2	0.32
Methanogens	Carbon	11.25	0.05*
	Nitrogen	10.41	0.01*
	pH	6.9	0.15
	Oxygen	5.6	0.20
Methanotrophs	Carbon	7.7	0.11
	Nitrogen	10.9	0.01*
	pH	7.7	0.14
	Oxygen	13.3	0.01*

*=significant

the study by Pittl et al. (2010). Our results support the assumption that tank bromeliads may provide an ideal habitat for microorganisms in the canopy of neotropical forests (Goffredi et al. 2011b).

Beside differences in microbial community size between tank slurry and soil samples (Pittl et al. 2010), the taxonomic compositions also differed. These differences may be explained by habitat-specific conditions. We found a 8-fold higher carbon and 6-fold higher nitrogen content in the tank slurries than in the adjacent soil samples. Also water conditions between bromeliads and nearby located ponds were previously described to be different. For instance, some aquatic invertebrates, like the bromeliad ostracod (*Elpidium bromeliarum*) were only found in tank bromeliads (Lopez et al. 2009), which differ in terms of specific composition compared to nearby ponds and swamps (Little and Hebert 1996).

Table 3 Methane oxidation rates in five bromeliad tank slurries of *W. gladioliflora*

Bromeliad	CH_4 Oxidation rate (μg gdw h^{-1})
A	24.9
B	28.0
D	42.8
F	62.4
H	25.1
Average (± SE)	36.6 ± 7.2

SE = standard error

Consistent with our hypothesis, our results indicate that each tank bromeliad creates a unique island in the canopies with respect to its microbial community. Within one individual bromeliad, however, microbial community structure showed a negligible variability probably due to mixing events caused by rainfall or animal interactions. The microcosms of tree holes face episodic mixing of nutrients due to stemflow (Walker et al. 1991). This may also be possible for tank bromeliads. The income of rainwater probably causes turbulences and therefore results in uniform conditions within the whole plant and in the establishment of similar microbial communities in each leaf axil. Sampling time points or sampling intervals may play a major role in order to detect controls on microbial community composition in tank bromeliad wetlands. In contrast to other studies, which lasted up to years (Carrias et al. 2001; Goffredi et al. 2011b; Carmo et al. 2014), our study captured only a snapshot in time. However it is to be emphasized, that due to different water availabilities the microbial community composition in tank slurry can dramatically change even within days (Brandt et al. 2014). Here, all tank slurry samples were taken within three days to limit further variations (e.g. seasonal changes, changes in water availability) revealing that the microbial community composition did not differ between leaf axils of a single plant but between individual plants although they belonged to the same species and grew in the same habitat patch. The bacterial and methanogenic communities showed thereby a more distinct clustering between tank slurries than the archaeal or methanotrophic communities did.

The archaeal community was dominated by a 393-bp TRF. In former studies this TRF was assigned to the hydrogenotrophic methanogens *Methanomicrobiales* or *Methanocellales* (Brandt et al. 2014; Martinson et al. 2010). The methanotrophic community was dominated by a 245-bp TRF (Supplementary Fig. S1d) and did not differ in its composition when one individual was excluded from the statistical analysis. In a previous study this 245-bp TRF was assigned to type-II methantotrophs consisting of various genera of methanotrophic bacteria (e.g. *Methylocystis*, *Methylosinus*; Lüke 2010). DNA fingerprinting, although a reliable and highly

reproducible technique and frequently used for soils and marine environments, shows here a limited phylogenetic resolution for the methanotrophic community. Nevertheless, TRFLP analysis for the bacterial, archaeal and methanogenic community seem to be sufficient to reveal significant differences in community compositions between tank slurries. A high bacterial diversity and variability were also observed for other tank bromeliad species (Carmo et al. 2014) and habitats within tropical canopies. Differences in bacterial communities within and between tree species were reported by Lambais et al. (2006) investigating the phyllosphere from tree canopies of the Atlantic forests. This pre-existing variability of microbial organisms colonizing the tree phyllosphere may in turn favor the development of unique microbial community compositions in tank bromeliads since incoming leaves serve as nutrient input. Lambais et al. (2006) explained the variations in community compositions due to different leaf ages, location in the canopy, light incidence, and microclimate conditions that influence the leaf environment. Also the individual location of a bromeliad may therefore play an important role and influences the receipt of water, leaf litter, nutrient, light radiation (Guimaraes-Souza et al. 2006) and interactions with other organisms (e.g ants; Blüthgen et al. 2000). A habitat-associated effect was also observed investigating terrestrially and epiphytically grown tank bromeliads. Bromeliads associated with different forest strata, and their constrained environmental characteristics affected methane cycling (Martinson et al. 2010) and may indicate differing methanogenic community clusters within forest canopies. Several other studies further indicated that the morphology of a bromeliad species can influence the microclimatic characteristics as well as the community of insects and other macroinvertebrates in the tank of these plants (Jabiol et al. 2009; Marino et al. 2013). However, the influence of plant morphology was reduced in this study, since we used solely tank bromeliads of the same species and of similar size. Already at this level microbial community composition differed between plants.

To determine chemical factors that potentially influence the microbial community in the tank we investigated tank properties such as carbon, nitrogen and fatty acid concentration as well as pH and oxygen concentration which in turn may be influenced by the receipt of leaf litter, its quality/quantity and precipitation. Along with our hypothesis we clearly observed differences in pH, carbon, nitrogen and oxygen concentration between plant slurries and especially carbon content revealed to be a major determinant for the bacterial, archaeal and methanogenic community composition. Carbon availability was already identified as one of the main drivers of microbial community structure in soil (Fierer et al. 2003) and Lopez et al. (2009) assumed that occasional organic matter inputs can induce a eutrophic condition in tank-bromeliads. In a previous study by Goffredi et al. (2011b) it was shown that pH has an effect on the bacterial community composition and that a low water content and therefore an increased oxygen exposure, affected the bacterial and archaeal community composition in tank bromeliad slurry (Brandt et al. 2014; Brandt 2015). In the present study, the microbial communities were further sorted along nitrogen concentrations in the tank slurry. Nitrogen is a limited factor for tank bromeliads. The plants receive nitrogen by mineralization of organic material from the canopy, from atmospheric sources (Stewart et al. 1995), by interactions with animals (Davidson and Epstein 1989; Leroy et al. 2013) or probably by microbial N_2 fixation (Brighigna et al. 1992).

Finally, we assume that the place where a tank bromeliad develops is important, since carbon and nitrogen concentration of tank slurries are parameters which solely depend on (incoming) detritus as the basal resource and affect the inhabiting microbial communities.

Nevertheless, the explained variation of microbial communities was relatively low in this study. On the one hand this can be due to environmental factors that were not measured, such as water volume which was shown to affect invertebrate diversity and the archaeal and bacterial community composition and the pathway of methane formation in tank bromeliads (Dézerald et al. 2014; Brandt et al. 2014; Brandt 2015). On the other hand there is probably a large stochastic component connected with the ephemerality of the plants and dispersal dynamics (Farjalla et al. 2012; Jocque and Field 2014). Tank bromeliads are dynamic systems with high emigration rates since many inhabitants of bromeliads can fly or use vectors that are mobile (Lopez et al. 2002; Jocque and Field 2014).

Conclusion

According to our hypothesis we could show that each individual tank bromeliad plant created a unique habitat with a distinct microbial community characterized by

the structure of four different genes (bacterial 16S rRNA gene, archaeal 16S rRNA gene, *mcrA*, *pmoA*). By contrast, these microbial communities were relatively homogenous within each individual plant. Our study confirmed the presence of methane-producing microorganisms. It further more demonstrated the presence of methanotrophic bacteria (characterized by *pmoA*) and of methane oxidation activity. Hence, a full methane turnover seems to be possible in tank bromeliad slurries.

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