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Effects of FPOM size and quality on aquatic heterotrophic bacteria

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ABSTRACT

Mechanical and biological processing in aquatic systems converts coarse particulate organic matter (CPOM) into fine particulate organic matter (FPOM). Other sources of particles with different size classes include flocculated dissolved matter, algae and soil particles. The relative magnitudes of these inputs are influenced by the degree of allochthony of a lake or stream. The size-reactivity hypothesis, formulated for dissolved organic matter, postulates that bacterial degradation rates are higher with high-molecular-weight fractions than with low-molecular-weight fractions. In this study, we investigated the effect of particle size on degradation of POM and on freshwater bacterial communities. We generated leaf-derived particle size classes of the same age (same diagenesis status) but differing in quality (maple and beech leaves). Contrary to our expectations, we found a strong effect of particle size and no significant effect of substrate quality on community respiration which decreased at smaller particle size, on C:N ratios which declined with particle size, and on δ^{15}N which showed a decreasing trend (though not significant) at smaller particle size in beech leaves. By contrast, bacterial community structure and ∂^{13}C values responded mainly to particle quality. Bacterial biomass, estimated by qPCR, was affected by complex interactions between particle size and quality. These findings open an unanticipated perspective on the size-reactivity hypothesis for particulate organic matter.

1. Introduction

Freshwater systems are often dominated by allochthonous carbon sources, mainly derived from vascular plants. This coarse particulate organic matter (CPOM, >1 mm) is transformed into fine particulate organic matter (FPOM, <1 mm–>25 μm), dissolved organic matter (DOM, <0.25 μm) and CO_2 by the combined effects of leaching, microbial maceration and degradation, physical forces and macroinvertebrate feeding (e.g., Cummins and Klug, 1979; Suberkropp and Klug, 1980; Allan and Castillo, 2008). FPOM is an important potential food source for collector invertebrates, i.e., filter-feeders and gatherers, which feed on suspended and benthic particles, respectively (Merritt and Cummins, 2006).

In freshwater ecosystems, the size of POM between 10 and 500 μm was positively correlated with organic content and nutritional quality, i.e., larger particles contained less lignocellulose and more protein, simple carbohydrates, lipids and microbial biomass (Peters et al., 1989). On the other hand, the C:N ratio generally increases from wood to leaves to FPOM (Findlay et al., 2002). Lower C:N ratios are taken as indicators of greater nutritional quality (Danger et al., 2016) as more nitrogen is available per biomass. In addition, there is a pronounced shift from predominantly fungal biomass on the largest particle categories (leaves) to predominantly bacterial biomass on smaller particles (Findlay et al., 2002); however, fungal diversity was greater on smaller particles (Wurzbacher et al., 2015). Microbial respiration is generally lower on smaller than on larger particles (Yoshimura et al., 2008, 2010). While the basic steps in leaf (CPOM) breakdown and physical and biological processing have been well characterized, very little information exists on the reactivity (decomposability) of smaller particles and their microbiological parameters. A further complication is the fact that sources other than deciduous leaves, such as algal biofilms and flocculation of dissolved organic substances, contribute to the pool of particles.

Amon and Benner (1996) proposed a size-reactivity continuum model, which relates smaller size of dissolved organic molecules to reduced reactivity. It is based on the rationale that smaller sized OM is more aged than larger OM and therefore more refractory. Subsequent studies have focussed on the diagenesis, composition, and size of dissolved OM in aquatic ecosystems and refined this hypothesis with emphasis on the effect of age and/or quality (Mannino and Harvey, 2000; Sannigrahi et al., 2005; Kaiser and Benner, 2009;

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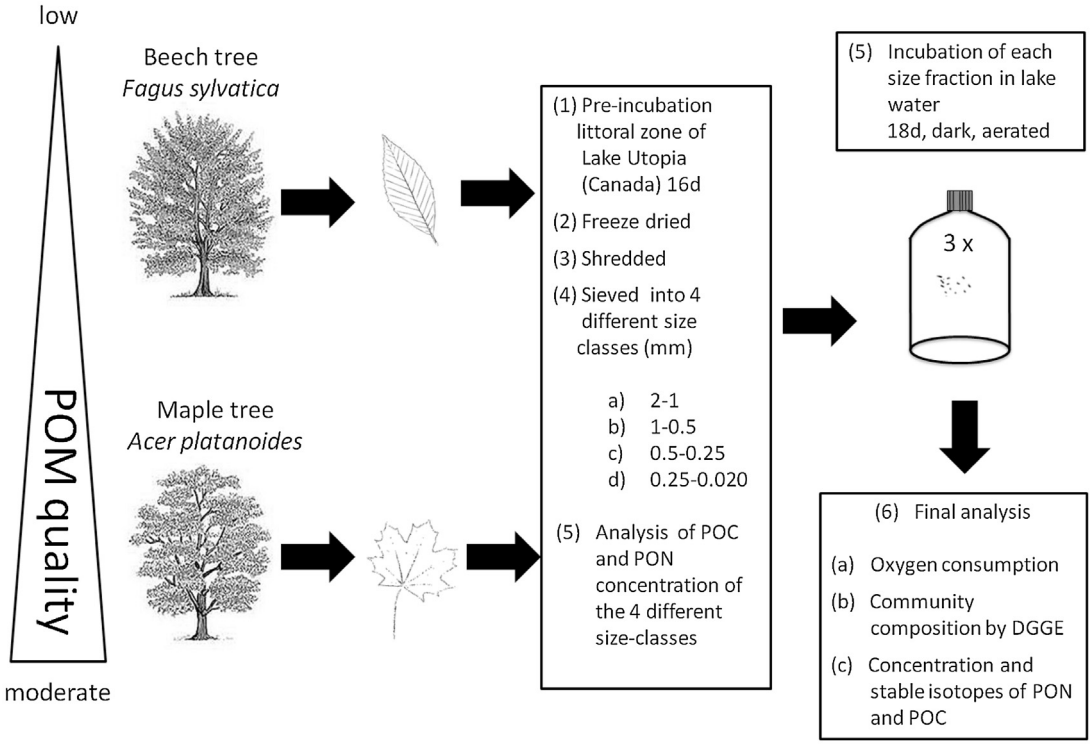

Fig. 1. Generation of leaf particles of different size classes.

Walker and McCarthy, 2012; Zigah et al., 2014) while disregarding size, which is considered a natural consequence of the sequential, selective degradation of larger OM (Benner and Amon, 2015). While this model has been validated for marine systems, several factors are likely to modify the size-reactivity concept in freshwaters, among them the presence of allochthonous carbon sources or minerals (Hedges et al., 2000; Fischer et al., 2002; Zigah et al., 2014), and the higher turnover rate of algal-derived low molecular weight DOC (Mannino and Harvey, 2000; Zigah et al., 2014). Particulate matter has rarely been considered under the size-reactivity hypothesis, and in most cases only to demonstrate that DOM has lower reactivity and quality, and a different composition than POM (e.g. Sannigrahi et al., 2005; Walker and McCarthy, 2012). The allochthonous carbon introduced by leaf litter fall is initially of the same age and biological maceration introduces particles with a short processing history into freshwater systems. There is little information on the impact of different POM size classes on microbial metabolism and attached species composition. To elucidate this, we investigated the effect of FPOM size and quality on microbial community respiration and bacterial composition. We incubated particles differing in quality (derived from maple or beech leaves) and four size classes in the laboratory. Leaves were preincubated in a lake to remove soluble leachate and invertebrate feeding as potential confounding variables. Since this largely cancels age (particle diagenesis) as predictor variable, we hypothesized differences in carbon consumption between (i) the two examined leaf species to be based on their quality and (ii) among size classes due to an altered surface to volume ratios of the particles (Peters et al., 1989). We expected facilitated bacterial colonization and respiration on smaller particles with a larger surface to volume ratio. Differences in carbon consumption might effect changes in microbial community structure and (iii) we expected to find differences in decomposition of particles due to the selective removal of carbohydrates and proteins detectable by analyzing the stable isotope signature of POC and PON of the different leaves and size classes.

2. Methods

2.1. Generation of particles

Senescent leaves from two individual trees were collected in the fall of 2010, air-dried and stored until needed. Two species were evaluated, based on their decomposability: 1) Maple (*Acer platanoides*) leaves exhibiting a moderate rate of decay and 2) European beech (*Fagus sylvatica*) leaves with a slow rate of decay (Bärlocher, 2005). To initiate particle generation, we preincubated the leaf litter in packs of 15 g from August 31 to 15 September, 2011, in the littoral zone of Lake Utopia, a slightly humic clearwater lake in eastern Charlotte County, New Brunswick, Canada, with total phosphorus values below 10 μg/l (Carr et al., 2004). From July–September 2011, the lake was characterized by pH 6.8 – 7.2, TP < 10 μg/l, TN < 0.3 mg/l (below detection limit), TOC < 8.5 mg/l, ammonium 0–5 μg/l, chlorophyll a 2.5 – 5.1 μg/l, phytoplankton biomass < 0.3 mg/l (Eastern Charlotte Waterways Inc., http://www.ecwinc.org/reports/). This exposure allowed leaching and initial microbial colonization but largely excluded invertebrate colonization and feeding. Upon collection, leaves were rinsed and freeze-dried. Leaf veins were removed prior to shredding in a kitchen blade granulator (Moulinette, Germany). Shredded leaves were sieved through a stack of 4 metal filters with different mesh-sizes, yielding 4 FPOM fractions (fraction 1: 2–1 mm (1.5 mm); fraction 2: 1–0.5 mm (0.75 mm); fraction 3: 0.5–0.25 mm(0.375 mm), fraction 4: 0.25–0.020 mm (0.135 mm)). The last step was achieved by applying a gauze with a mesh-size of 20 μm. The steps in the preparation of the particles are illustrated in Fig. 1.

2.2. Characterization of particles

Initial C and N concentrations of the 8 combinations of particle size and leaf species were determined in 3 replicates following recovery from the lake, using an elemental analyser (Euro EA 3000;

HEKAtech GmbH, Wegberg, Germany) after grinding leaves in a mixer mill (MM 2000, Retsch, Haan, Germany) and placing 2 mg per replicate in tin capsules.

Each combination of particle size and leaf species was incubated separately in triplicates for 18 days at 17 °C with constant aeration. Incubations took place in 2 l glass bottles in the dark with 46 (±1) mg/l leaf particles in 2 liters of prefiltered (250 μm) lake water (estimated total carbon input per incubation as 50% of dry mass: 46 mg). Oxygen consumption was measured on days 5, 8, 11, and 14 with at least 12 h per measurement following the manufacturer's instructions (SensorDish reader with glass vessels, *PreSens* Precision Sensing GmbH, Regensburg, Germany). Respiration followed a unimodal pattern with a maximum on day 11 (1.8 mg/day) and minima on day 5 (0.8 mg/day) and 14 (0.9 mg/day). For each measurement, 5 ml were removed without replacement. Consumed oxygen was transformed into mg of respired carbon with a respiration quotient of 1 (Berggren et al., 2012) and a molar conversion factor from oxygen to carbon of 0.375. The mean total carbon consumption was estimated by averaging the consumption of all four sampling dates followed by extrapolation to 18 days. After 18 days of incubation, all particles were harvested by vacuum filtration. For end-point determination of POC and PON concentration, 250 ml were filtered through a 5.0 μm precombusted silver filter (Sterlitech, Kent USA). Stable N and C isotope ratios (δ^{15}N-PON, δ^{13}C-POC) and PON and POC concentration were measured by means of flash combustion in a Carlo Erba EA 1108 at 1020 °C in a Thermo Finnigan Delta S mass-spectrometer. Silver filters containing particle samples were trimmed, sectioned and then loaded into tin capsules and pelletised for isotopic analysis. The stable N and C isotope ratios measured for each sample were corrected against the values obtained from standards with defined N and C element and isotopic compositions (International Atomic Energy Agency IAEA: IAEA-N1, IAEA-N2, NBS 22 and IAEA-CH-6) by mass balance. Values are reported relative to atmospheric N_2 (δ^{15}N) and VPDB (δ^{13}C-Vienna Peedee belemnite). The analytical precision for both stable isotope ratios was ±0.2‰. Calibration material for C and N analysis was acetanilide (Merck, 1.00011).

2.3. DNA analyses

For DNA analyses, 500 ml water were filtered through a 5.0 μm polycarbonate filter (Millipore) after 18 days of incubation. DNA was extracted from the filters with the PowerSoil MoBio Kit. DNA was subjected to quantitative PCR to detect molecular abundances of bacterial ribosomal gene copies. For bacteria, we used the PerfeCTa Sybr Green Mix (Quanta Bioscience) in an CFX96 instrument (BioRad) with the primer system BACT1369f and PROK1541r (Suzuki et al., 2000) and conditions as described earlier (Wurzbacher et al., 2012) *Escherichia coli* DNA was used for standard dilutions. The amplification-efficiency was 99% (the optimum range is between 90 and 110%). All qPCR reactions were further evaluated by standard melt-curve inspection. Retrieved data were evaluated by arbitrarily setting 1 ng of reference strain (*E. coli*) to 1 billion copies. All copy numbers were normalized to sample DNA and log2 transformed.

Bacterial community composition was assessed by denaturing gradient gel electrophoresis (DGGE) in a Dcode system (BioRad). We amplified bacterial 16 S genes by primer pair 341f-GC and 907r with PCR and DGGE conditions as described in Allgaier and Grossart (2006). Gels were inspected visually for presence and absence of bands and samples were transformed into a binary matrix.

2.4. Statistical analyses

ANOVAs and regressions were performed with JMP Mac Version 10.0.2 (www.jmp.com), with particle size and leaf species as nominal variables. Slopes and intercepts of regressions were compared with an extra sum-of-squares F test (Motulsky and Christopoulos, 2004).

Binary matrices resulting from the presence/absence of bands in DGGE were used for non-metric-multidimensional scaling (NMDS) in R based on the Jaccard index. The NMDS ordination (stress = 0.12) was overlayed with a pruned average clustering (UPGMA) and a confidence interval (0.95) around groups centroids (vegan package in R 2.14.1; Oksanen et al., 2011).

3. Results and discussions

3.1. FPOM size and quality and its effect on bacterial respiration

We tested the effects of FPOM size and quality, represented by particles derived from two leaf species (Fig. 1), on bacterial community respiration and composition. Initial C:P ratios after lake exposure are listed in Table 1. They were consistently higher with maple despite its greater decomposability, confirming that the rate of leaf decay is influenced by complex interactions between total carbon, nitrogen, lignin, and cellulose (Talbot and Treseder, 2012). Within the examined range of particle sizes, the C:N ratio declined with particle size except for size class 1–2 mm in maple leaves (Fig. 2). This decline probably reflects varying proportions of cuticle, epidermis and mesophyll. Carbon concentrations were significantly influenced by leaf (ANOVA, $p < 0.0001$), but not by particle size (ANOVA, $p = 0.50$), nor by their interaction (see above $p = 0.13$).

On average, the estimated total carbon consumption over the entire incubation time of 18 days of the various particle fractions and leaf species was 20.5 mg (46% of initially added carbon) with considerable variation between size-fractions (Fig. 2A). Total carbon consumption was positively related to FPOM particle size and differed slightly, but non-significantly ($p = 0.17$), between the two leaf species (Fig. 2A; regression for combined data: $Y = 16.78 + 5.48 \times X$, $r2 = 0.56$, $p < 0.0001$; Table 2). This was also mirrored in the carbon balance, which points to an increasing carbon enrichment with decreasing particle size.

By contrast, the C:N ratios at the end of the experiment differed significantly between the leaf species but no longer between particle sizes, though there was an apparent decline towards smaller sizes (Fig. 2B, Table 1). ∂13C values were significantly influenced by leaf species, but not by particle size (Fig. 2C, Table 2), and approached significance for ∂15N values (Fig. 2D, Table 2).

Total respiration was positively correlated with particle size, i.e., smaller particles presumably decomposed more slowly (Fig. 3). This is in contrast to our hypothesis (ii) that smaller particles with a larger surface to volume ratio may facilitate bacterial colonization and respiration. Our observations are however supported by two studies that also reported lower respiration on the smallest FPOM (Yoshimura et al., 2008, 2010). The authors related the size of particles to differences in lignin, cellulose and phosphorus concentrations. Similarly, Peters et al. (1989) report the lowest bacterial production on the smallest particles. Jackson and Vallaire (2007), who compared the decomposition rate of FPOM below and above a 0.25 mm threshold for benthic FPOM, reported an opposite trend, and attributed this to different carbon sources (terrestrial versus algal carbon) for the two particle fractions. Yoshimura et al. (2010) also documented high respiration rates on algal particles. However, the particles in these studies were either naturally occurring "aged" particles or selectively processed (gut passage) particles. In our study, we largely excluded ageing effects or differential pre-treatment of the particle fractions by exposing the intact leaves to a conditioning phase before particle formation. We were therefore able to selectively examine the effects of size and substrate type. We also minimized the effect of the particles' origin

Table 1
C:P ratios of particles after lake exposure (average of 3 replicates, ± SD). 2-Way ANOVA ($p < 0.0001$ for size, substrate and size × substrate). Tukey-Kramer multiple comparson tests ($p \leq 0.05$, separately for two substrates, values with same superscript do not differ significantly). Carbon budget calculated from the initial added carbon, endpoint carbon measurements and estimated total respired carbon per particle size fraction.

Particle size	Maple	Beech	Carbon budget maple (Δ initial carbon) [mg/l]	Carbon budget beech (Δ initial carbon) [mg/l]
1	$67.4^{b} \pm 0.5$	$58.1^{a} \pm 0.6$	49.7 (−3.7)	49.8 (−3.8)
1–0.5	$71.1^{a} \pm 1.0$	$55.7^{b} \pm 0.6$	47.8 (1.8)	41.6 (4.4)
0.5–0.25	$68.2^{b} \pm 0.9$	$54.6^{b} \pm 0.3$	37.2 (8.8)	36.4 (9.6)
0.025–0.02	$62.0^{c} \pm 0.7$	$50.1^{c} \pm 0.4$	35.2 (10.8)	37.4 (8.5)

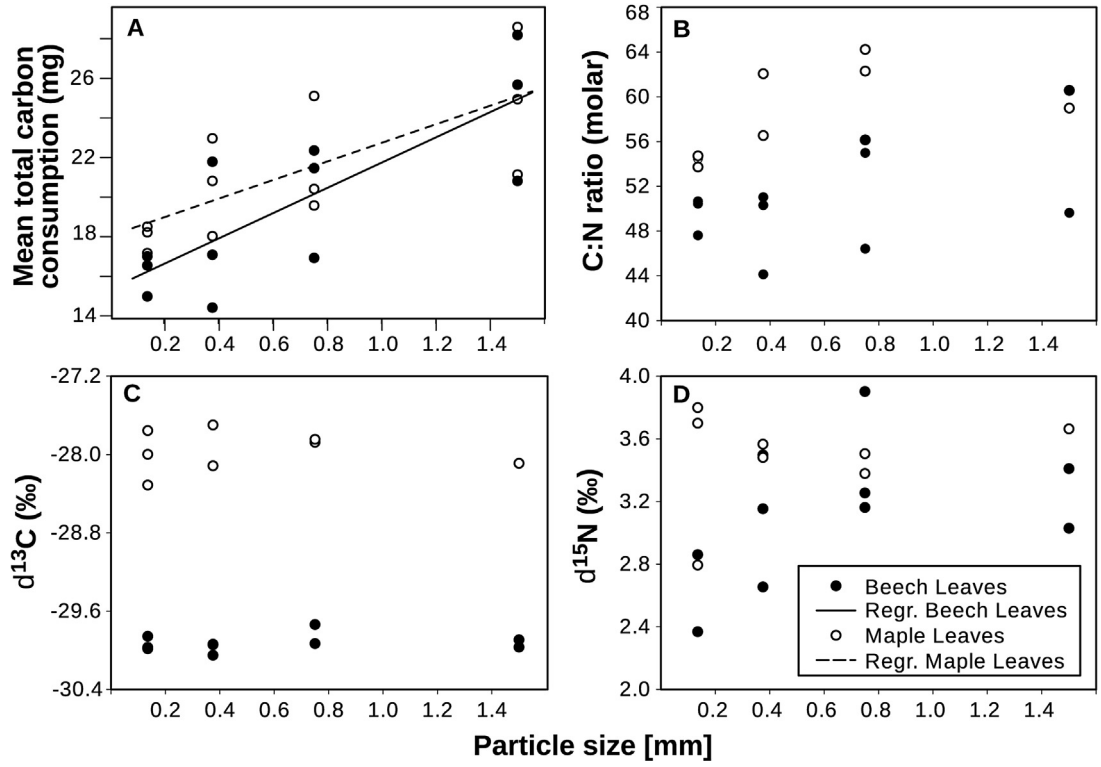

Fig. 2. Total carbon consumption of leaf-litter derived particles (A), their final C:N ratio (B) and their isotopic carbon (C) and nitrogen (D) signatures. The results of statistical analyses are summarized in Table 1.

Table 2
Summary of statistical analyses (2-Way ANOVAs, data from Fig. 2A–D, and from Fig. 4).

Dependent variable	Data from	Source	df	SS	F	p
C consumption	Fig. 2A	Species	1	13.8	1.65	0.22
		Size	3	197.9	7.88	0.002
		Species × size	3	6.2	0.25	0.86
C:N ratio	Fig. 2B	Species	1	472.4	5.93	0.03
		Size	3	119.4	0.501	0.69
		Species × size	3	63.2	0.27	0.85
$\partial^{13}C$	Fig. 2C	Species	1	23.5	1191.3	<0.0001
		Size	3	0.04	0.73	0.55
		Species × size	3	0.03	0.39	0.76
$\partial^{15}N$	Fig. 2C	Species	1	0.71	4.70	0.055
		Size	3	0.51	1.13	0.38
		Species × size	3	0.39	0.86	0.49
qPCR data	Fig. 4	Species	1	0.02	0.30	0.59
		Size	2	0.06	0.37	0.78
		Species × size	3	1.31	7.82	0.002

by using exclusively allochthonous leaf material, which is generally considered as more recalcitrant than algal-derived material. Interestingly, the differences in C:N ratios between the two leaf species (Fig. 2B, Tables 1 and 2), which might explain variations in decomposition rates, had only a marginal effect on total respiration (Figs. 2 A, 3). The initially lower C:N ratio in the smaller-sized particle fractions was maintained during the incubation period, indicating preferential carbon loss through processes such as bacterial colonization and subsequent nitrogen immobilization (Thornton and McManus, 1994; Fellerhoff et al., 2003),

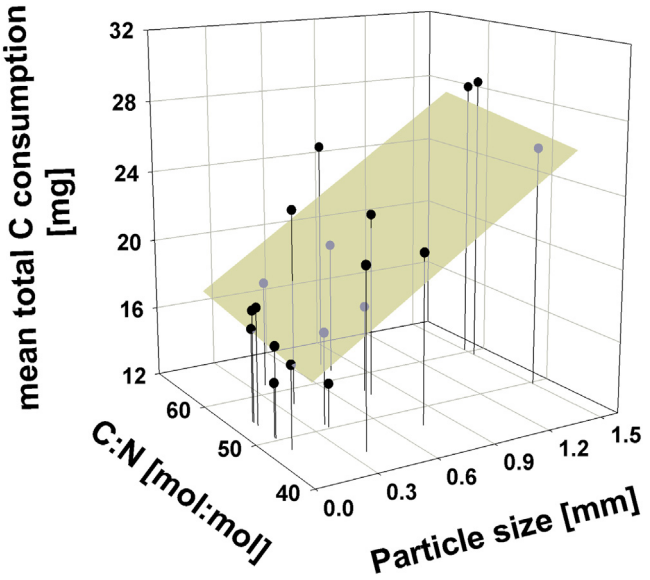

Fig. 3. Microbial respiration vs. C:N ratios and particle size. Multiple regression: Consumption = 12.1 + 0.07 × (C:N ratio) + 7.10 × (Size); $R^2 = 0.701$, $p < 0.001$.

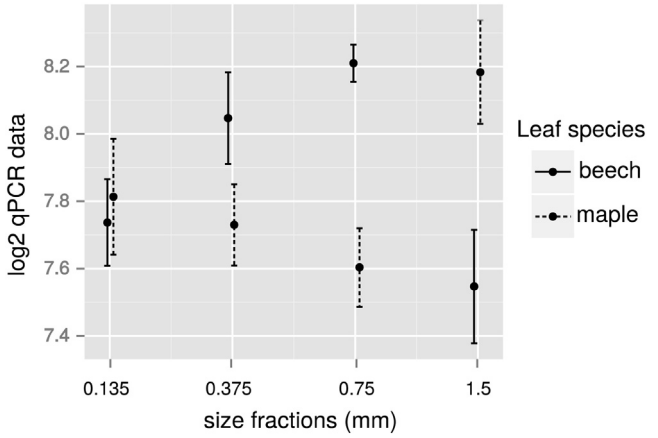

Fig. 4. qPCR results of the four particle size fractions. Means ± standard error.

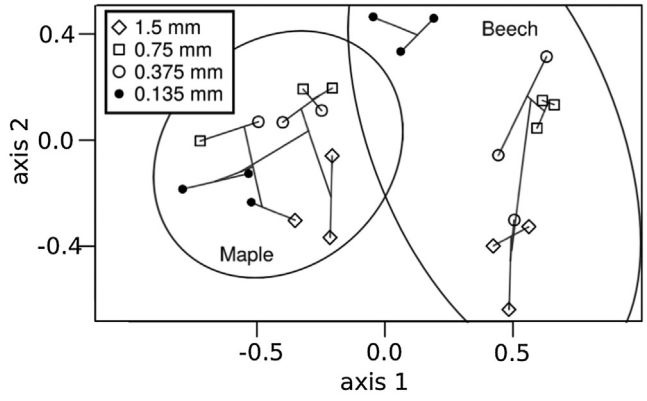

Fig. 5. NMDS of the bacterial communities on leaf-derived particles based on DGGE patterns. Ellipses indicate the standard deviation around the group centroid. Connecting lines indicate the underlying average clustering of the samples by distances.

increased carbon loss through microbial respiration or mineralization (Fellerhoff et al., 2003), or preferential removal of carbohydrates (containing only C, H and O) during the first 100 days of decomposition (Fogel and Tuross, 1999). We therefore conclude that respiration was influenced by the quality of the particles. However, we could not detect any relationship between total respiration and C:N, which was confirmed by linear and rank-based correlation analyses ($p > 0.1$ within leaf species or globally). This supports our initial hypothesis (ii) that the measured difference in functionality was mainly caused by particle size, which overrode the effects of leaf-species (both effects are summarized in Fig. 3). We should note, however, that the lake water used in this experiment was nutrient poor with low phosphorus values below 10 μg/l. Microbial metabolism was likely phosphorus limited and our conclusions need to be verified for nutrient rich environments.

To elucidate this quality/size effect more closely, we investigated the stable isotopes of carbon and nitrogen (Fig. 2C, D; Table 2). Overall, the stable isotopic signature of $\delta^{13}C$ showed more negative values in beech than in maple (−29.93 ± 0.08‰ and −27.96 ± 0.2‰, respectively, Fig. 2C) and confirms the difference in substrate quality. There was no significant correlation between $\delta^{13}C$ and particle size ($p = 0.72$). A decrease in $\delta^{13}C$ is usually due to (microbial) removal of carbohydrates and proteins (Hedges et al., 1988; Harvey et al., 1995), leading to an enrichment of lignin compounds. The stable $\delta^{13}C$ values suggest that size selective leaching of dissolved organic matter (DOC) after particle generation had no major impact on the experiment. However, differences in stable isotopes might already have been present at the start of the experiment (not determined) and the carbon balance (Table 1) suggests that selective leaching is also a possibility.

$\delta^{15}N$ values of the two leaf species did not differ from each other at the conventional significance level (Fig. 2D; $p = 0.055$). Correlations between particle size and $\delta^{15}N$ were not significant ($p = 0.91$), but a trend was detectable in beech leaves with decreasing $\delta^{15}N$ at smaller particle size (Fig. 2D). For $\delta^{15}N$, changes usually occur during assimilation of dissolved inorganic nitrogen (DIN) by bacteria. The isotopic depletion in $\delta^{15}N$ from large to small particles (~0.6‰) during incubation of beech leaves might be the result of selective remineralization of heavier nitrogen isotopes (Currin et al., 1995) and assimilation of nitrogen by the bacterial community during decomposition (Caraco et al., 1998). Taking into account C:N ratio and respiration data, we conclude that size does indeed determine the decomposition rate of leaf material, and that despite their higher surface to volume ratio smaller particles decay more slowly.

3.2. *Effect of FPOM Size and quality on bacterial abundance and community composition*

In addition to the chemical parameters, we determined changes in bacterial abundances and community compositions estimated by quantitative PCR (qPCR) and DGGE. Overall, leaf species did not significantly affect bacterial copy numbers, though there was a highly significant interaction between particle size and leaf species (qPCR data; Fig. 4, Table 2). Particles derived from the two leaf species produced opposing patterns, which points to differences in the composition of microbial communities due to interactions between particle size and quality. The greatest difference between leaf species in ribosomal gene copy numbers was found in the larger particles while copy numbers were almost identical on the smallest particles (200–20 μm). On beech but not on maple particles there was a significant difference between copy numbers on the four particle sizes (Fig. 4, Beech: One-way ANOVA, p 0.025; Maple: One-way ANOVA, $p = 0.09$). However, similar to the ATP measurements by Peters et al. (1989), there was no linear correlation between particle size and microbial biomass.

We further evaluated the composition of the microbial communities by DGGE and analyzed the data by non-metric multidimensional scaling (NMDS, Fig. 5). The two leaf species harbour

distinct microbial communities (Fig. 5, PERMANOVA: $r^2 = 0.41$, $p = 0.0005$, 2000 permutations). This was well reflected by the particles' chemical properties ($\delta^{13}C$: $r^2 = 0.87$, N: $r^2 = 0.65$ with $p < 0.001$, respectively; and C with $r^2 = 0.49$ with $p < 0.01$; ordination based model fit), supporting hypothesis (iii) that there is a qualitative difference between particle from the two leaf species.

Particle size clearly structured the bacterial community only in beech leaves and separated the smallest particles from the rest (Fig. 5). Thus, we conclude that a) the two leaf species were colonized by different microbial communities and b) that there is a connection between particle size and bacterial communities on low quality FPOM. It is well known that substrate quality shapes bacterial communities (e.g. Docherty et al., 2006), however, for submerged leaf litter (CPOM) this effect has been reported as being absent or weak (Dieter et al., 2013). In our study we clearly see differences between the two leaf species and thus FPOM colonization seemed to be influenced by the substrate type. In addition, we see a clear effect of particle size on bacterial community composition on beech, which seems to be absent on the higher quality substrate (maple). A possible explanation is that smaller particles have a higher surface area covering fewer resources. If those resources are of lower quality, the microenvironment might become (i) more selective and/or nutritionally poorer while (ii) being spatially restricted. This may inhibit the establishment of interacting (e.g., commensalistic, mutualistic) species pairs and groups. Further taxonomic and enzymatic analysis may clarify the underlying mechanisms of this species-specific size effect.

4. Conclusions

Our results demonstrate that particle size influences bacterial activity and community composition while particle quality modulates this effect. At first glance, this aligns with the size-reactivity continuum model of Amon and Benner (1996), formulated for dissolved organic matter (Benner and Amon, 2015). However, our results consider particulate OM and add a physical factor (size) to the rationale. We excluded diagenesis (in the sense of chemical transformation via enzymatic action) in our experiment but nevertheless confirmed an apparent size-reactivity relationship, in the sense that the total carbon turnover was reduced in smaller particles. Furthermore, a clear structuring of the bacterial communities on the two leaf species driven by particle size was visible in the NMDS plots with a separation of the smallest particles on low quality FPOM. We conclude that at least for POM, physical size in addition to diagenesis plays a role. A selection mechanism connected to size is suggested by the observed changes in the bacterial community: decreasing particle size may select against a fully functional microbial network essential for breaking down complex leaf materials. This size effect might be especially important after the autumnal leaf fall, when the ages of the particle size classes are initially fairly similar. Additional investigations are needed to clarify the reactivity of the smallest particles and to formulate a general model which includes age and the POC-DOC transition.

Our results may be of interest in technical applications regarding the decompostion of minced plant biomass in liquid bioreactors .

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