

Direct and Indirect Effects of Millipedes on the Decay of Litter of Varying Lignin Content

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1. Introduction

Millipedes are considered to be important organisms involved in decomposition, both for their direct feeding on detritus and their indirect effects on microbial activity. Hanlon (1981a, 1981b) suggested that fragmentation of leaf litter by soil fauna increases microbial biomass by increasing leaf surface area and diminishing pore sizes. The passage of litter through the gut of macroarthropods, such as millipedes, can help in the establishment of soil bacteria (Anderson & Bignell, 1980; Hanlon, 1981a, 1981b; Tajovsky et al., 1991; Maraun & Scheu, 1996). The presence of millipedes has been shown to increase the decomposition of litter as well as increase growth of seedlings (Cárcamo et al., 2001). In a beech forest, Bonkowski et al. (1998) also found that the presence of millipedes significantly increased the decomposition of litter, much more so than endogeic earthworms. The presence of millipedes has also been found to greatly increase the release of litter nutrients into the soil, especially calcium and nitrates (Pramanik et al., 2001). Millipedes are selective about what leaves they eat (Lyford, 1943; Kheirallah, 1979; Cárcamo et al., 2000). The chemical composition of leaf litter, especially the lignin and nitrogen content, can greatly affect soil fauna populations, although this effect is not clear for millipedes (Tian et al., 1993). Van der Drift (1975) estimated that in temperate areas millipedes are responsible for ingesting 5–10 percent of the annual leaf litter fall and Cárcamo et al. (2000) estimated that a single species of millipede consumed 36 percent of the annual leaf litter in a British Columbian Cedar-Hemlock forest. Tropical studies have also found a large influence of millipedes on decomposition (Tian et al., 1995). In a Tabonuco forest in Puerto Rico, Ruan et al. (2005) found that millipede density explained 40 percent of the variance in leaf litter decomposition rates, while soil microbial biomass explained only 19 percent of the variance.

Millipedes make up a large part of the arthropod community on the forest floor in the Tabonuco forests of Puerto Rico. Richardson et al. (2005, pers. com.) found that diplopods in El Verde (a Tabonuco forest) constituted about 11.4 percent (73.09 mg dry/m²) of the microarthropod biomass, second only to Isoptera. In the same forest, we found that Stemmiulidae were the most abundant millipede with a density of ca. 22 individuals/m² (Murphy et al., 2008).

In this study, we use a microcosm approach to answer the direct (leaf fragmentation) and indirect (microbial biomass) effects of millipedes on the decomposition of leaf litter and how these outcomes are influenced by the substrate (litter) quality and the density of millipedes. We expect that higher the litter quality (lower lignin content) and the higher density of millipedes would result in more leaf area lost, decreased leaf mass remaining, and higher biomass of soil microbes. We used microcosms containing one of three litter species with varying lignin to nitrogen (L/N) ratios and three different densities of millipedes.

2. Methods

2.1 Site

Millipedes and soil for microcosms were collected from the Luquillo Experimental Forest, a subtropical wet forest located near the El Verde Field Station, (18°19' N, 65°45'W) in Río Grande, Puerto Rico in June 2006 (Fig. 1). Vegetation at this elevation (420m) is called Tabonuco forest after the dominant plant species *Dacryodes excelsa*.



Fig. 1. Millipedes and soil for microcosms were collected from the Luquillo Experimental Forest, a subtropical wet forest.

The mean annual air temperature in the Luquillo Mountains is 22.3°C (Brown et al., 1983) and the mean annual precipitation is 3525 mm with rainfall distributed more or less evenly throughout the year (Garcia-Martinó et al., 1996). The dominant soil orders in the Luquillo Experimental Forest are Ultisols and Inceptisols (Brown et al., 1983). Soil series in the Tabonuco forest vary according to topography: *Humatas* on ridges, *Zarzal* or *Cristal* on slopes, and *Coloso* in valleys (Johnston, 1992; Soil Survey Staff, 1995).

2.2 Experimental design

The microcosms consisted of clear plastic containers, measuring 19 cm x 13 cm x 9 cm (area ca. 0.025m²), with mesh tops (1.5mm) to keep millipedes within and to exclude other

organisms (Fig. 2). The microcosms were kept in a covered room at El Verde Field Station that was open to the environment laterally to keep conditions as close to field conditions as possible. One milliliter of distilled water was sprayed into the microcosms each day to mimic mean daily rainfall. Each microcosm contained 115g of sieved (1.19mm) uniform soil from the Tabonuco forest.



Fig. 2. The microcosms consisted of clear plastic containers, with mesh tops to keep millipedes within.

We used three different leaf species (*Dacryodes excelsa*, *Manilkara bidentata*, and *Rourea surinamensis*) (Fig. 3), one species per microcosm and three densities of millipedes (0, 2, and 5 individuals) with three replicates of each of these treatments, collected at two weeks after setup (July 13, 2006) and four weeks after setup (July 27, 2006). Additionally, we had an initial collection (June 29, 2006) of nine microcosms (3 litter species, 3 densities of millipedes) that were returned to the laboratory immediately after set up to establish handling loss and dry mass relationships (e.g., González & Seastedt, 2001). In total, there were 63 microcosms used in the experiment. Millipedes used in the microcosms were all from the Order Stemmiulida, Family Stemmiulidae (Fig. 4). Millipedes in the density of two individuals had a combined fresh weight of approximately 0.04g and those in the density of five individuals had a combined net weight of about 0.08g. Leaves were obtained from El Verde Tabonuco forest and air dried. They were then cut into 3 x 3 cm squares and 5 grams of leaf squares were added to each microcosm. The three plant species were chosen because of their frequent occurrence in the Tabonuco forest and their leaves having a range of lignin to nitrogen ratios, with similar percents of nitrogen and varying lignin amounts (Table 1). Other plant species in this forest have a range of L/N ratios from 6.2 to 62.5 (Zalamea & González, unpublished data).

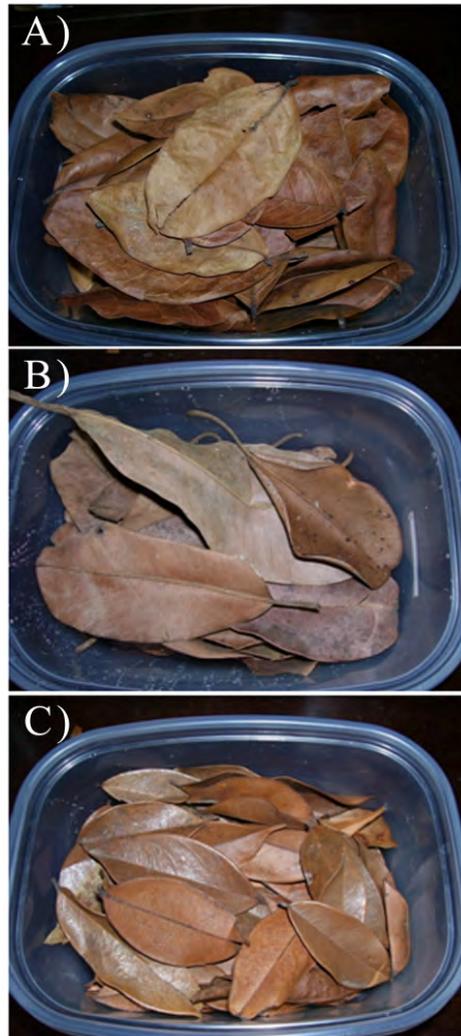


Fig. 3. Three leaf species with varying lignin to nitrogen (L/N) ratios were used in the experiment, A) *Dacryodes excelsa*, B) *Manilkara bidentata*, and C) *Rourea surinamensis*.

Plant Species	% Nitrogen	% Lignin	L/N ratio
<i>Dacryodes excelsa</i>	0.81	22.89	28.26
<i>Manilkara bidentata</i>	0.72	25.17	34.96
<i>Rourea surinamensis</i>	0.76	31.13	40.96

Table 1. Initial leaf chemistry of *Dacryodes excelsa*, *Manilkara bidentata*, and *Rourea surinamensis* (Zalamea & González, unpublished data).



Fig. 4. Millipedes used in the microcosms were all from the Order Stemmiulida, Family Stemmiulidae.

2.3 Measurements

At each collection, fifteen leaf squares were chosen at random and flattened in a plant press overnight. Leaf area for each of these 15 squares was then measured using an area scanner (LI-3100 Area Meter, Li-Cor, Inc.), taking three measurements of each square and averaging them. All leaves from the microcosms were oven-dried at 60° C and weighed. The fresh weights of millipedes were obtained initially and when microcosms were collected. The final oven dried weights of millipedes were also measured. In addition, when microcosms were collected all of the soil was removed, pH measured (with a 1:1 KCl to soil ratio) and inorganic nitrogen content (in the form of NO_3^- and NH_4^+) extracted with KCl and measured with a Rapid Flow Analyzer RFA-305 (Alpkem Corporation, Clackamas, Oregon). Soil microbial biomass C was estimated by the substrate induced respiration (SIR) method (Lin & Brookes, 1999) calibrated for the study area (Zalamea & González, 2007), using an ER-10 Columbus Instruments respirometer, and calculated from the CO_2 evolved (Anderson & Domsch, 1978).

2.4 Data analysis

The general linear model procedure was used for multivariate analyses of variance (MANOVA) to test the effect of litter species, millipede density, and collection time on the leaf area remaining, percent leaf mass remaining, millipede weight, soil pH, soil nitrate, ammonium concentrations, and soil microbial biomass (SPSS Inc., 2001, v. 11.0.1). Post-hoc tests were performed to determine significant differences among the litter species, millipede density, and collection time for each of the dependent variables, using Student-Newman-Keuls (SNK); $\alpha = 0.05$. Additionally, Pearson's correlations (2-tailed) were calculated

between the initial leaf chemistry (%N, %L, L/N ratio) and the leaf area remaining, percent leaf mass remaining, microbial biomass, and soil inorganic nitrogen. All statistical analyses were performed using SPSS Inc., 2001, v. 11.0.1.

3. Results

The leaf area remaining and the percent leaf mass remaining were significantly affected by all three independent variables (litter species, millipede density, and the time of collection) (Table 2). The percent of leaf mass remaining was significantly the lowest for *Dacryodes excelsa*, and the highest for *Manilkara bidentata* (Fig. 5A, Fig. 6).

The leaf area remaining was significantly different for all three litter types, following a pattern of significantly less leaf area remaining for the lower L/N ratio species (*D. excelsa*) and significantly the most area remaining for the highest L/N ratio species (*Rourea surinamensis*) (Fig. 5B). The highest density of millipedes (five individuals) had significantly less mass remaining than the litter from those microcosms without millipedes (Table 3).

The leaf area remaining was significantly less in the last collection, indicating that after four weeks the leaves had been significantly fragmented, while after two weeks the leaf area was not significantly different from the initial collection. The percent leaf mass remaining decreased significantly from one collection to the next (Fig. 6). At the two week collection Tabonuco leaves had significantly less percent of leaf mass remaining when millipedes were not present, and at the four week collection *R. surinamensis* leaves had significantly higher percent of leaf mass remaining when millipedes were not present (Fig. 6).

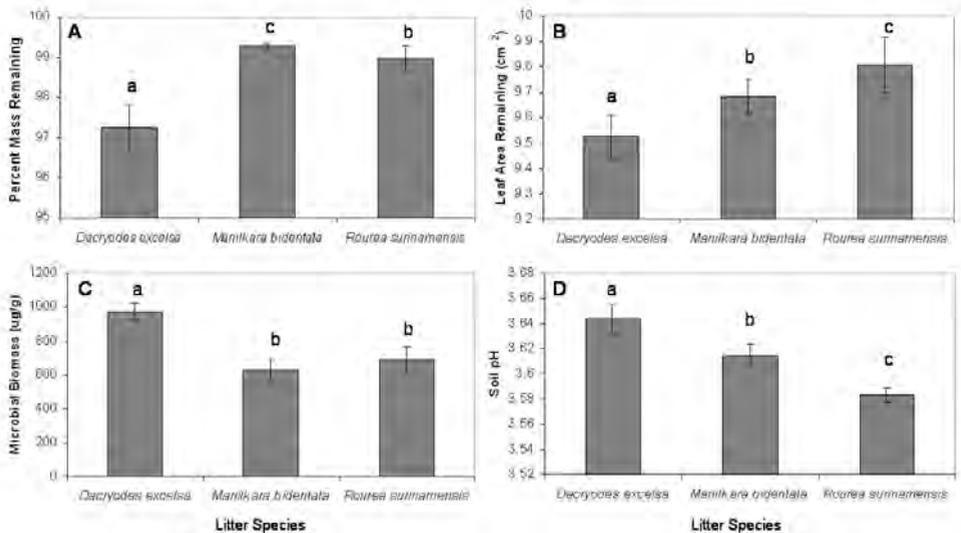


Fig. 5. A) The percent leaf mass remaining, B) leaf area remaining, C) microbial biomass, and D) the soil pH in microcosms containing *Dacryodes excelsa*, *Manilkara bidentata*, or *Rourea surinamensis* leaves. Significant differences are indicated by different letters (Student-Neuman-Keuls tests; $\alpha=0.05$). The standard error of the mean is indicated with error bars.

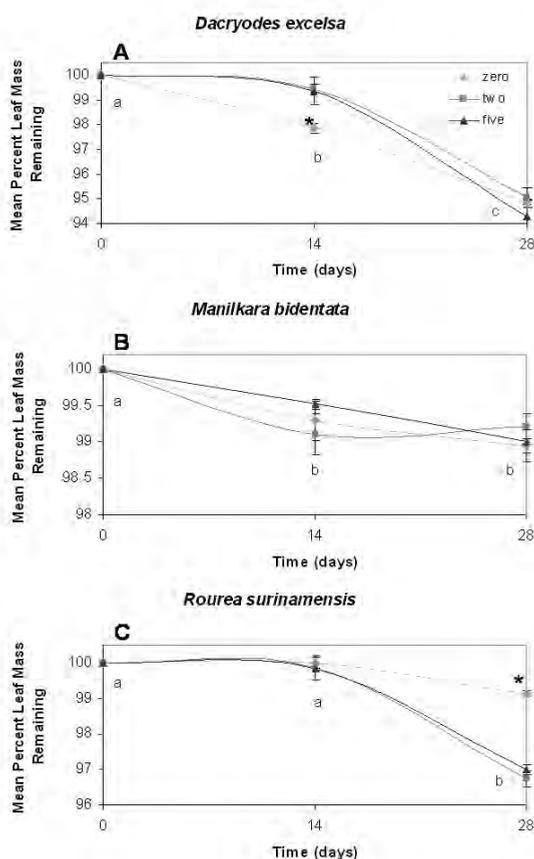


Fig. 6. The mean percent of mass remaining over time for each of the densities of millipedes (light gray for zero, dark grey for two, black for five) in microcosms with A) *Dacryodes excelsa*, B) *Manilkara bidentata*, or C) *Rourea surinamensis*. Different letters in the same graph represent significant differences among collection times. Asterisks (*) indicate a significantly different effect of the indicated millipede density at this collection time (Student-Neuman-Keuls tests; $\alpha = 0.05$).

The initial leaf chemistry was significantly correlated with the leaf area remaining, percent leaf mass remaining, soil microbial biomass, and soil nitrate, but not soil ammonium. The percent nitrogen in leaves was negatively correlated with the percent leaf mass remaining ($P < 0.01$) and positively correlated with the soil microbial biomass ($P < 0.01$). The initial percent nitrogen in the leaves was not significantly correlated with either soil nitrate or soil ammonium. The initial percent lignin was positively correlated with the leaf area remaining ($P < 0.05$) and the percent leaf mass remaining ($P < 0.05$) and negatively correlated with soil microbial biomass ($P < 0.05$) and soil nitrate ($P < 0.01$). The L/N ratio was also positively correlated with the leaf area remaining ($P < 0.05$) and the percent leaf mass remaining ($P < 0.01$) and negatively correlated with soil microbial biomass ($P < 0.01$) and soil nitrate ($P < 0.05$).

Source	Variable	df	F	P	Power
Litter Species (L)	LAR	2	24.75	0.00	1.00
	PMR	2	48.13	0.00	1.00
	Soil pH	2	144.12	0.00	1.00
	Microbial Biomass	2	19.70	0.00	1.00
Millipede Density (M)	LAR	2	5.08	0.01	0.79
	PMR	2	8.62	0.00	0.95
	Soil pH	2	42.58	0.00	1.00
	Microbial Biomass	2	1.10	0.34	0.23
Time (CT)	LAR	2	115.86	0.00	1.00
	PMR	2	189.54	0.00	1.00
	Soil pH	2	220.18	0.00	1.00
	Microbial Biomass	2	130.46	0.00	1.00
L x M	LAR	4	5.02	0.03	0.94
	PMR	4	16.43	0.00	1.00
	Soil pH	4	8.32	0.00	1.00
	Microbial Biomass	4	1.04	0.40	0.29
L x CT	LAR	4	5.94	0.00	0.97
	PMR	4	43.99	0.00	1.00
	Soil pH	4	169.31	0.00	1.00
	Microbial Biomass	4	15.87	0.00	1.00
M x CT	LAR	4	6.35	0.00	0.98
	PMR	4	7.49	0.00	0.99
	Soil pH	4	18.93	0.00	1.00
	Microbial Biomass	4	0.62	0.65	0.18
L x M x CT	LAR	8	6.30	0.00	1.00
	PMR	8	5.67	0.00	1.00
	Soil pH	8	51.43	0.00	1.00
	Microbial Biomass	8	0.74	0.65	0.29

Table 2. Effect of litter species (*Dacryodes excelsa*, *Manilkara bidentata*, and *Rourea surinamensis*), millipede density (zero, two, and five), and the collection time (zero, fourteen, and twenty-eight days) on the leaf area remaining (LAR), percent leaf mass remaining (PMR), soil pH, and microbial biomass. Degrees of freedom (df), F and P values, and Power for MANOVA are presented. Statistics were performed using SPSS Inc., 2001, v. 11.0.1.

Density of Millipedes	Percent Leaf Area Remaining	Percent Leaf Mass Remaining	Millipede Weight lost (g)	Microbial Biomass ($\mu\text{g/g}$ of soil)	pH
Zero	96.677 ^a	98.692 ^b	.000 ^b	692.808 ^a	3.633 ^b
Two	97.125 ^a	98.567 ^{ab}	.003 ^b	572.203 ^a	3.605 ^a
Five	97.345 ^a	98.271 ^a	.022 ^a	615.774 ^a	3.604 ^a

Table 3. The mean percent leaf area, percent mass remaining, millipede weight lost, microbial biomass, and soil pH for each of the densities of millipedes in microcosms. Different letters in the same column signify significant differences among densities (Student-Neuman-Keuls tests; $\alpha = 0.05$).

Soil pH was significantly different among microcosms with different litter types, with *R. surinamensis* soil being the most acidic and Tabonuco (*D. excelsa*) soil being the least acidic (Fig. 5D). The presence of millipedes also caused the pH to slightly but significantly decline (Table 3). The soil pH at the final collection was significantly less acidic than in the first two collections (Table 2). Soil inorganic nitrogen was significantly lower in *R. surinamensis* soil than for the other two litter types (Figs. 7A-B). Soil nitrate did not vary through time (Figs. 7C). While, soil ammonium increased significantly from one collection to the next through time (Figs. 7D).

Neither form of inorganic nitrogen varied significantly with different densities of millipedes. Microbial biomass in the soil of Tabonuco microcosms was significantly higher than for the other two litter types (Fig. 5C). The microbial biomass did not differ depending on the density of millipedes. Additionally, microbial biomass significantly decreased throughout the experiment (Fig. 8). The millipedes lost weight as the experiment continued. Millipedes lost significantly more weight when they were in the highest density (Table 3).

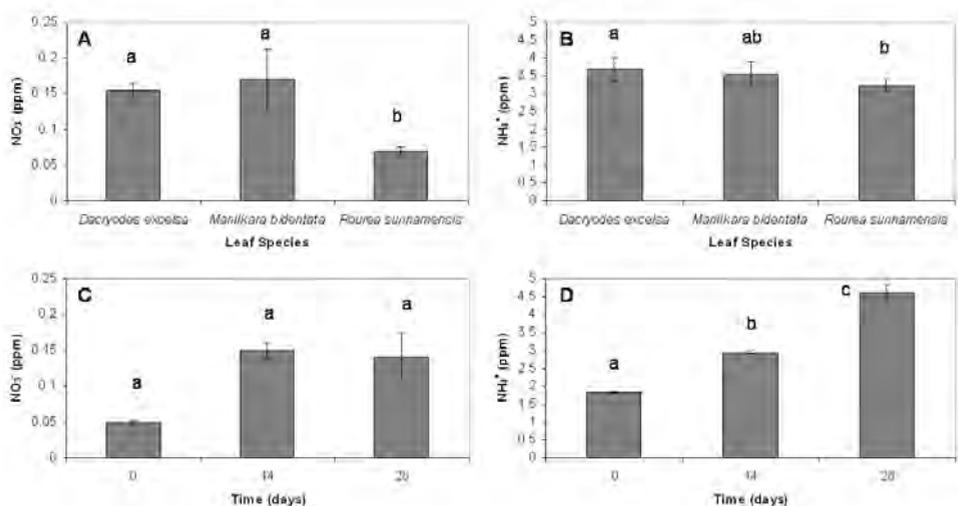


Fig. 7. (A) Soil nitrate and (B) ammonium content for microcosms containing leaves of *Dacryodes excelsa*, *Manilkara bidentata*, and *Rourea surinamensis*. (C) Soil nitrate and (D) ammonium measurements over the three collection times. Standard error of the mean is represented by error bars. Different letters in the same graph represent significant differences among collection times (Student-Neuman-Keuls tests; $\alpha = 0.05$).

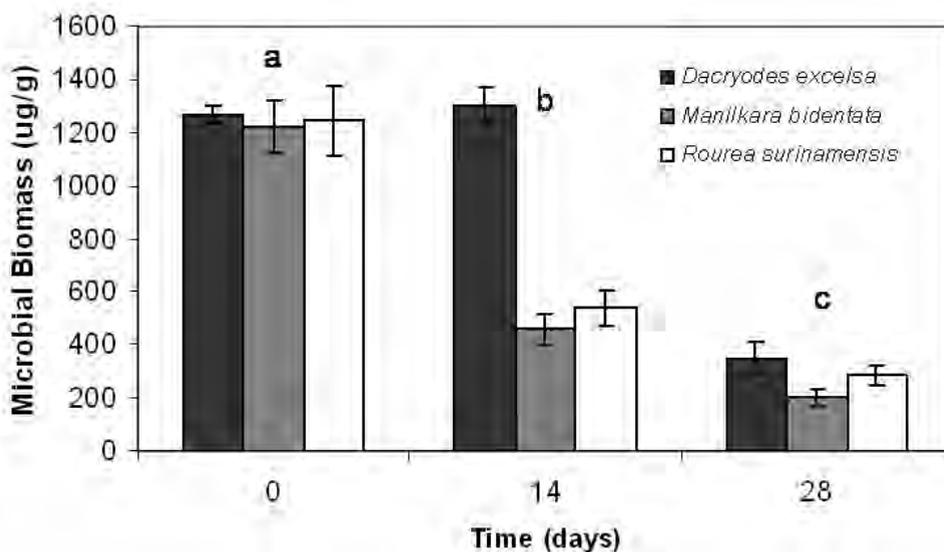


Fig. 8. The microbial biomass in microcosms containing leaves of *Dacryodes excelsa*, *Manilkara bidentata*, and *Rourea surinamensis* at three collection times. Different letters indicate significant differences among each of the collection times (Student-Neuman-Keuls tests, $\alpha=0.05$) of the overall microbial biomass.

4. Discussion

Millipedes directly impacted the decomposition of leaf litter in this microcosm study. The millipedes significantly fragmented the leaf litter by the fourth week. Direct fragmentation was affected by the substrate quality, with the higher lignin content (higher L/N ratio) leaf species having more leaf area remaining and percent mass remaining. The direct impact of millipedes also depended on the density of millipedes, with the higher density significantly decreasing the mass remaining of the leaves. Although this impact differed by leaf species: the low lignin content Tabonuco leaves lost more mass without millipedes at one of the collections, while the high lignin content *R. surinamensis* leaves lost more leaf mass with millipedes at the last collection (Fig. 6). These findings were similar to those of Tian et al. (1995), who found a decrease of percent mass remaining of leaves with very high lignin (47 percent) and C/N ratios by millipedes and earthworms in the field conditions. They concluded that microbes could independently breakdown the higher quality litter, so that the macrofauna influence was not as significant for higher quality leaves (<12 percent lignin content) (Tian et al., 1995), a probable explanation for our results as well. We did find that the high-quality *D. excelsa* soil had by far the greatest microbial biomass, suggesting that both the millipedes and microbes were influencing the breakdown of Tabonuco leaves. If we had continued this experiment for longer we might have seen more dramatic results in leaf fragmentation. For example, Coûteaux et al. (2002) found that after 198 days in a microcosm, a single millipede turned over on average nearly half of the five grams of pine litter given into frass when microcosms were kept at intermediate temperatures (15-32° C).

In the present study, leaf chemistry affected the fragmentation of leaves. Leaf species with a high percent of lignin and high L/N ratios were correlated with higher leaf area remaining and percent mass remaining. In the tropics, Aerts (1997) found that lignin-to-nitrogen ratios of leaf litter were the best chemical predictors of litter decomposition. In general, tropical leaf litter has lower L/N ratios with a mean of 24.2, while temperate regions average a value of 32.0, and 29.4 for Mediterranean regions (Aerts, 1997); our values ranged from 28 to 40 (Table 1).

Indirectly, the millipedes could have influenced the microbial biomass of the soil, though this influence is harder to tease apart than their direct effects. In this study, through time microbial biomass diminished, which could indicate that the millipedes had a negative effect on the microbes, although this argument is weakened by finding that adding millipedes to microcosms and increasing the density of millipedes had no significant effect on the microbial biomass. Our results suggest that millipedes had no effect on microbial biomass but that leaf chemistry did: percent leaf lignin and leaf L/N ratios were negatively correlated with soil microbial biomass. Kaneko (1999) found decreased microbial biomass when millipedes were present in a laboratory experiment, but when the same experiment was done in the field (an oak forest in Japan) millipedes had no effect on the microbial biomass. When Hanlon & Anderson (1980) introduced diplopods and isopods into microcosms they at first increased microbial biomass, but then returned to control levels after twelve days. We could have missed an initial increase in microbial biomass because our first collection was not until the fourteenth day. The decrease of microbes through time in this experiment could also be connected with the millipedes losing weight during the experiment, which seemed to be from the drier conditions of the microcosms because they did not hold water and humidity as well as their natural environment. We suggest that the effect of millipedes on microbial biomass be further tested under field conditions to confirm their impact.

The pattern of soil inorganic nitrogen could be explained in two ways. The first mechanism is by nitrogen leaching from the leaves. The soil under *Tabonuco* leaves, which had the highest percent nitrogen, also had the highest values of inorganic nitrogen while *R. surinamensis*, with the lowest percent nitrogen in their leaves, also had the lowest values of inorganic nitrogen in the soil. This pattern was not significant when a Pearson's correlation between the initial leaf chemistry and both soil inorganic nitrogen measures was calculated. Secondly, *Tabonuco* microcosms had the highest biomass of microbes, indicating a more active mineralization and faster turn over rate in the *Tabonuco* soil, which would release inorganic nitrogen into the soil. In this experiment, the latter seems to be the more probable source of inorganic nitrogen. Many authors have found significant increases in inorganic nitrogen from soil arthropods, due to their excrement, indirect efforts on microbes, and/or fragmentation of litter (Ineson et al., 1982; Persson, 1989; Setälä, et al., 1990; Teuben & Verhoef, 1992; Cárcamo et al., 2001; Pramanik et al., 2001; etc.), but this did not seem to be a factor in our results as millipede density had no significant effect on either soil ammonium or nitrate.

Both the leaf chemistry and the millipede density affected the soil pH: the less lignin and the absence of millipedes resulted in less acidic soil. Anderson & Domsch (1993) showed that microbial biomass decreases in more acidic soil, further influencing the decomposition. The distribution of microarthropods can also be influenced by soil pH (Hågvar, 1990;

Klironomos & Kendrick, 1995; Dlamini & Haynes, 2004). We found the highest microbial biomass in the less acidic Tabonuco soil (the least %L and L/N ratio) and this species also had significantly the least leaf area remaining and percent mass remaining, but the presence of millipedes also affected these results by decreasing the percent mass remaining.

5. Conclusion

We conclude that millipedes can impact leaf litter decomposition both directly and indirectly, but the extent of their effect depends on their density and the quality of the substrate (leaf lignin content). Directly, it is clear that millipedes fragment litter, which in some cases has been shown to indirectly increase microbial biomass (e.g. Hanlon, 1981a; 1981b). We did not find an increase but a decrease of soil microbial biomass over time, yet microbial biomass was not affected by the presence of millipedes, suggesting that other factors might have been driving this trend. Millipedes are not the only arthropods influencing decomposition, and their interactions with other organisms in their natural environment could affect the extent of their influence. Notwithstanding, we suggest that the results of this microcosm experiment be applied to millipedes in their natural setting, further confirming millipedes as important components of soil ecosystems and nutrient cycling.

6. Acknowledgements

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