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Effects of leaf quality and colonization time on the abundance of bacteria in an experimental design

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ABSTRACT. Small rivers, henceforth streams, depend on organic matter (nutrients and energy) from riparian vegetation. The quality of such allochthonous debris is determinant for the transformation of organic matter compounds, where the bacterial community has a crucial role in the final decomposition of the substrate. During bacterial colonization, debris with higher concentration of nutrients (more palatable) is prioritized, which accelerates the process. This study investigated the effects of leaf palatability of two native trees on bacterial colonization (abundance) over time, through a laboratory experiment that lasted 60 days. Values of C, N, P, C:N, C:P, polyphenols, tannins, lignin, lignin:N and leaf toughness of both species were compared. Bacterial abundance was higher in species with higher nitrogen values, although they had higher leaf toughness and more polyphenols, which differs from studies indicating that high leaf toughness represents low nutritional quality. The colonization time did not influence bacterial abundance. Therefore, processes degrading riparian vegetation and reducing nutritional quality can affect local decomposition, decreasing bacterial abundance.

Keywords: streams; atlantic forest; laboratory experiment; decomposition; microbial ecology.

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Introduction

In streams, allochthonous organic matter (OM) is a pivotal source for the trophic web, making heterotrophic metabolism more prevalent (Esteves & Gonçalves, 2011). This OM from riparian vegetation falls directly into streams, or is carried by wind, flooding and rainfall, which displace material from soil into streams. Abundance of this vegetation affects the energy flow by inhibiting primary production through shading. Hence increasing the relative importance of allochthonous OM (Wallace, Eggbert, Meyer, & Webster, 1997).

Decomposition of riparian vegetation in streams is an important ecosystem service, which consists of the reduction and transformation of complex compounds into simple molecules through physical, chemical and biological processes (Farjalla, Marinho, & Esteves, 1999). Conventionally, the decomposition process is characterized by three phases: 1. Leaching, first phase, in which there is considerable loss of leaf mass, removal of water- and lipo-soluble compounds; 2. Microbial colonization, when there is potentiation of chemical modifications due to enzymes of microorganisms, increasing nutritional quality and palatability for the next phase; and, 3. Fragmentation, the action of leaf abrasion, originated from the consumption of OM by invertebrates, essentially the fragmenters (Webster & Benfield, 1986).

Succession of organisms throughout the decomposition phases is strongly related to the nutritional quality of OM. In addition, the chemical quality and exposure time of the leaf litter to the stream flow influence the OM decomposition rate. This occurs due to the release of soluble compounds over time (König, Suzin, Restello, & Hepp, 2008). Leaf litter decomposition may be slower according to nutrients present in water. This impasse is caused by the scarcity of nutrients related or not to the toughness of OM, which results in a lower microbial colonization (Gonçalves, Graça, & Callisto, 2007). On the other hand, the decomposition rate can be accelerated in leaf litter with high concentration of water-soluble compounds, such as phenols, nitrogen or phosphorus (Gonçalves, Rezende, Martins, & Gregório, 2012). This phenomenon is known as

palatability, when leaf litter with the highest concentration of nutrients is prioritized during colonization by aquatic organisms, consequently accelerating the decomposition rate. Therefore, secondary compounds and high concentration of lignin and cellulose can slow or even inhibit the decomposition of riparian leaf litter, since few organisms have enzymes capable of degrading them (López-Rojo et al., 2018).

Bacteria are vital for leaf litter decomposition because of their role in the latter stages of this process on the substrate, remaining adhered throughout all stages and benefiting from the metabolism of other decomposers, either by the present compounds or by the changes made in the leaf parenchyma (Findlay et al., 2002; Artigas, Gaudes, Munoz, Romaní, & Sabaster, 2011). Moreover, this group represents a significant portion of the total biomass in leaf litter decomposition (Baldy, Chauvet, Charcosset, & Gessner, 2002). Also, in tropical streams, bacteria are important in the early decomposition stages, metabolizing proteins and easily assimilated molecules, until the massive replacement with fungi (Gonçalves, França, Medeiros, Rosa, & Callisto, 2006).

Thus, this study aimed to investigate how leaf palatability affects bacterial colonization over time in streams, using a laboratory experiment to control most variables affecting leaf litter decomposition and colonization by these microorganisms. Finally, two hypotheses were tested: 1) leaf litter decomposition by microorganisms in streams is higher in the most palatable leaves, so that bacterial abundance is higher in the most palatable leaves; and 2) bacterial abundance, in streams, increases over time in which plant species.

Methods

Selection and characterization of plants

Two species that naturally occur in the riparian vegetation of the Atlantic Forest were selected: *Cariniana estrellensis* (Raddi) Kuntze (Lecythidaceae) and *Nectandra cuspidata* Nees (Lauraceae) (a.k.a. jequitibá-branco and canelinha, respectively). Both trees are common in the study area, Northern Paraná, Southern Brazil, with high relative abundance and high contribution of leaf litter to water bodies (Souza, Kawakita, Slusarski, & Pereira, 2009). Although *C. estrellensis* and *N. cuspidata* are native tree species, they may diverge in their chemical and nutritional composition, which means they may differ in palatability and consequently, leaf litter decomposition rates. Leaves were sampled using nets set in several stretches of riparian vegetation containing the selected species. These nets were kept open for a month and then leaves were collected and selected according to similar size and age. This sampling was carried out in the dry season, when trees regularly lose a high number of leaves due to water restriction. After sampling, aiming to slow initial leaching, leaves were packed in cardboard boxes to dry for over 72 hours at room temperature.

Experimental procedure

Sets of five discs (1 cm diameter) of each plant species, distinguished according to their palatability (more palatable and less palatable), were cut (with a cork cutter), weighed, and placed in litter bags (0.1 cm mesh size). Then, litter bags were disposed in trays with water, leaves and twigs from a stream in the region (selected because of its conserved features; 22°43'05.0"S 52°25'15.0"W or -22.718056 -52.420833), over 15 days, for colonization by microorganisms present in the stream (inoculum). Then, litter bags with leaf discs were placed in pots with approximately 400 mL stream's water. This water was filtered through a 15 µm mesh for maximum retention of algae and invertebrate but retaining the ambient natural microbiota, as much as possible. Then, pots were placed in trays in a refrigerated environment (19°C) and the water of trays was warmed using thermostats (22°C or 71.6ºF, annual average of the streams in the summer, when the experiment was conducted). Oxygenation was maintained with oxygen pumps. The experiment consisted of three pots (three replicates): in each pot, there were four sets of litter bags of each plant species (eight litter bags per pot, 3 replicates x 2 plants x 4 times = 24 samples). Litter bags were sampled (withdrawn) every 15 days, within a total period of 60 days (four samplings in total). Pots with water from the experiment were filled with stream's water throughout the experiment and were kept under controlled temperature. Leaf discs were used for bacterial abundance analysis. After removing bacteria from the discs, for counting, they were dried in a forced air oven for 48 hours and weighed to obtaining the leaf dry weight (DW). Given the influence of dissolved oxygen, pH and conductivity on the decomposition process speed (Griffith & Perry, 1993; Bjelke, 2005), these variables were monitored along the experiment by portable potentiometers, every three days.

Bacterial abundance

Leaf discs were frozen after each sampling date and subsequently stored in 5 mL 4% formaldehyde until analysis. For separation of bacteria from the leaf surface, an ultrasonic washer (40 W, 40 KHz, Eco-sonics, Unique, Brazil) was used, where the samples were sonicated for about one minute. After sonication, 400 µL samples were stained with 2 µL SYTO-13 dye (Molecular Probes; 2.5 µmol L-1 final concentration) for estimation of bacterial abundance, through a flow cytometer. Bacterial detection occurred with the plotting of lateral dispersion (SSC) against a FL1 (green fluorescence) and data was processed in the FlowJo version 10 *software*. Bacterial abundance was expressed in number of cells mg-1 leaf DW.

Data analysis

For evaluation of different leaf characteristics between plant species, t-tests were applied followed by Tukey post hoc tests for multiple comparison. The characteristics tested were: Carbon, Nitrogen, Phosphorus, Carbon: Nitrogen, Carbon: Phosphorus, polyphenols, tannins, lignin, lignin: Nitrogen and leaf toughness.

To test the first hypothesis, a paired t-test was applied to the data to check for differences in the abundance of bacteria between plant species with different palatability, assuming the bacterial abundance as a response variable and palatability as the predictor variable. To test the second hypothesis, one-way analysis of variance (ANOVA) was applied, assuming the abundance as response variable also and time as the predictor variable.

Data were investigated for normality (Shapiro Wilk test) and homoscedasticity (Levene test). All analyses were run in Statistica version 10.0 *software* (StatSoft), assuming a significance level of 5%.

Results

Leaves of the tree species presented similar values of carbon (C), nitrogen (N), C:N ratio, C:P ratio and polyphenols. Phosphorus (P), tannin and leaf toughness were higher in *C. estrellensis*, while lignin and lignin: N ratio were higher in *N. cuspidata* (Table 1).

Table 1. Physical and chemical characteristics of two native trees (*Nectandra cuspidata* and *Cariniana estrellensis*).

Physical and chemical characteristics	*N. cuspidata*	*C. estrellensis*	p
C (% DM)	50.91 ± 0.18	50.30 ± 0.38	0.221
N (% DM)	1.35 ± 0.08	1.50 ± 0.05	0.156
P (% DM)	0.014 ± 0.002	0.015 ± 0.002	**0.017**
Carbon: Nitrogen (molar)	44.28 ± 2.48	39.19 ± 1.30	0.144
Carbon: Phosphorus (molar)	9647.25 ± 1755.75	8401.27 ± 902.64	0.562
Polyphenols (% DM)	3.49 ± 0.09	5.08 ± 0.79	0.653
Tannin (% DM)	0.93 ± 0.01	1.16 ± 0.02	**<0.01**
Lignin (% DM)	53.38 ± 1.88	40.29 ± 0.36	**<0.01**
Lignin: Nitrogen (molar)	39.88± 3.22	26.90 ± 0.77	**0.017**
Leaf toughness (g)	180.81 ± 27.56	503.81 ± 78.41	**<0.01**

Values are presented as mean ± standard error. t-tests with $p < 0.05$ in bold.

The percentage of remaining DM was higher in *C. estrellensis* (k day^{-1} = 0.00531), in relation to *N. cuspidata* (k day^{-1} = 0.00231.) ($F(2,67) = 3.254$; $p = 0.044$). Leaf discs of *C. estrellensis* lost more than 25% DM, while those of *N. cuspidata* lost 10% (Figure. 1).

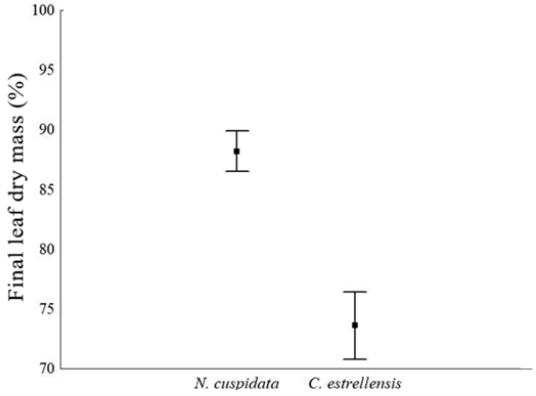

Figure 1. Remaining dry mass (%) over the experiment (60 days).

Bacterial abundance was estimated considering the plant species and over time (days), with water temperature kept at 22ºC. The highest abundance value was found in the first sampling, at 15 days, for leaves of *C. estrellensis*, followed by the third sampling, at 45 days, for the same species. The highest value for *N. cuspidata* was found in the first sampling (Table 2).

Table 2. Mean ± standard deviation of bacterial abundance present in leaf discs of *Nectandra cuspidata* and *Cariniana estrellensis* over time. Abundance values represent the number of bacterial cells mg-1 DW leaf discs.

Time (days)	*N. cuspidate*	*C. estrellensis*
15	30435.39 ± 2502.19	62103.69 ± 10906.33
30	25874.64 ± 2239.56	32507.46 ± 8420.19
45	22695.17 ± 5556.15	50411.71 ± 10031.84
60	24871.59 ± 3081.33	46105.99 ± 16048.81

Bacterial abundance was significantly different ($t(1.22) = 19.389$; $p < 0.01$) between the analyzed plant species, regardless of the sampling, with higher values in the most palatable species (Figure 2A). However, no significant differences were detected ($F(3,20) = 1.149$; $p = 0.354$) between samplings (Figure 2B).

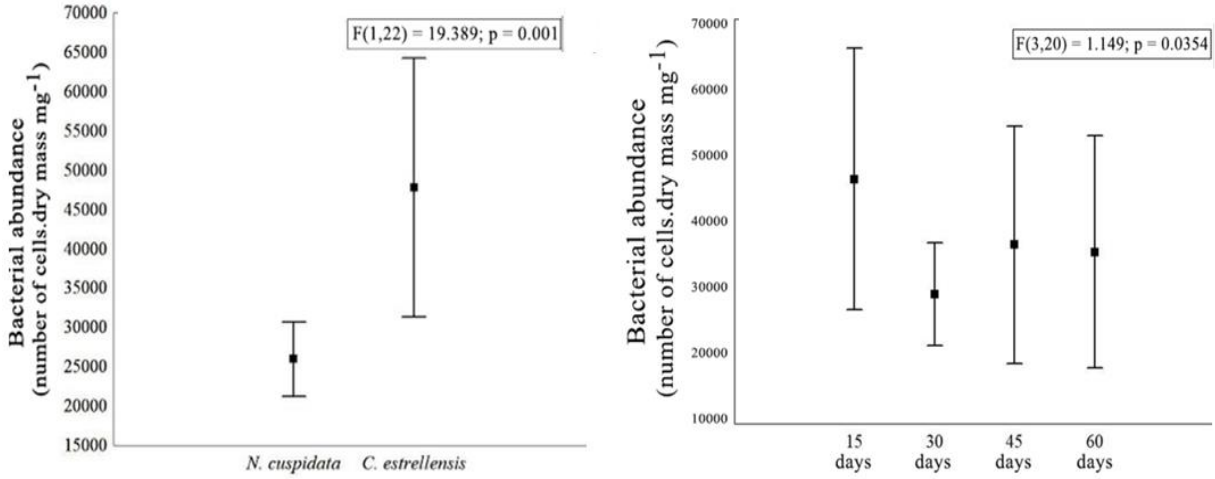

Figure 2. A: Mean ± standard deviation of bacterial abundance per native tree species (*Nectandra cuspidata*; *Cariniana estrellensis*); B: Mean ± standard deviation of bacterial abundance per samplings (days).

Discussion

Cariniana estrellensis showed the lowest values for the lignin: N ratio, which indicates a higher microbial activity and consequently higher abundance. This corroborates that the nutritional quality of leaves is directly related to their carbon and nitrogen rates, more specifically the ratio of these compounds (C: N) (Gonçalves et al., 2007). In the study by Gonçalves et al. (2007), the native species showed no significant differences from analysis of these compounds, however, diverged from lignin:N ratio. In addition, *C. estrellensis* presented a higher rate of tannins, corroborating Ardon and Pringle (2007), who reported that the influence of secondary compounds in leaves are less important than structural compounds on decomposition rates, since even with higher tannin values, *C. estrellensis* leaves had higher bacterial colonization.

Leaf litter of higher nutritional value is expected to be the one with lower leaf toughness, because of the microbial activity rate. However, our results showed the opposite, since *C. estrellensis* presented the highest leaf toughness values. Leaf toughness is determined by cellulose and lignin (polymers present in the plant cell wall), which are chemical compounds that can hinder access of decomposers to nitrogen in organic matter from riparian vegetation, thus these compounds interfere with decomposition by microorganisms and invertebrates. Nevertheless, this interference is low with bacterial activity, and more effective in inhibiting fungal activity (Talbot & Treseder, 2012). Therefore, although presenting higher values of leaf toughness and tannins, *C. estrellensis* was more palatable than *N. cuspidata*, which was corroborated by studies in tropical streams that recognizes leaf toughness as a strong controller of the litter breakdown rates (Ardon, Pringle, & Eggert 2009). So, our first hypothesis was accepted.

Our second hypothesis was not confirmed by the temporal variation of bacterial abundance, which proved to be oscillating. We expected that, over time, the abundance would increase over time, however higher values were observed in the first sampling, for both species, and the lowest values diverging between each species: *N. cuspidata* in the third sampling and *C. estrellensis* in the second sampling. Fluctuation in bacterial abundance may be related to different levels of palatability over time, influenced by polyphenols and tannins in water or bacteria, once dissolved they may be present in different compounds. Tannins are highly reactive and water-soluble, and can interact to a lesser or greater degree with molecules of bacteria and constitute reversible or irreversible complexes. There are at least three forms of tannins that inhibit microbial activity: i) to complex with bacterial proteins; ii) to act on the cell membrane; iii) to complex with metal ions (which would make them scarce for bacterial metabolism) (Mello & Santos, 2001). Preston, Trofymow, Niu, and Sayer (1997) suggest that, operationally, lignin residue analysis present structures derived from tannins, so lignin and leaf litter decomposition analyses may actually be reflecting the effects of tannins and other polyphenols on decomposition. Furthermore, the ability of tannins and polyphenols to bind to proteins predominates under conditions where there is no leaching and it can influence N mineralization and N immobilization, which contributes to oscillations in the decomposition rate by directly affecting N availability (Handayanto, Giller & Cadisch, 1997). In addition, there are many ways in which heterotrophic bacteria respond to resource availability. The availability of C and N, which vary throughout the year and seasons, may change the microbial community dynamics and its relationship with nutrients of the plant species (Montaño, Sandoval-Pérez, García-Oliva, Larsen, & Gavito, 2009).

The bacterial community plays a key role in the trophic chain, biogeochemical cycles and nutrient recycling, in tropical ecosystems. Studies suggested that bacterial communities may be more efficient than aquatic hyphomycetes in oligotrophic tropical streams (Medeiros et al. 2015). In tropical ecosystems, bacteria are more important at the early stages of decomposition because they metabolize proteins and sugars until the colonization of other microorganisms (Gonçalves et al., 2006). Furthermore, tropical forests have more abundant and productive bacterial communities, important for the rapid renewal of nitrogen (Vitousek & Sanford, 1986). Thus, their role in nutrient cycling in the food chain causes tropical ecosystems to be more efficient in these functions, being more productive. Moreover, tropical aquatic and terrestrial ecosystems are more dependent on these communities than temperate ecosystems (Alongi, 1994), however, they present weathered soils, depleted of nutrients by human activities, e.g. reduction of vegetation cover and converted into agricultural land, more frequently (Downing et al., 1999), which compromise the seasonality, availability and quality of allochthonous OM for the local biota. Also, the increasing temperature in aquatic ecosystems, which is predict by the intergovernmental panel on climate change (IPCC, 2014), may reduce the conditioning of the litter, that is more palatable to invertebrates, by reducing microbial colonization (Navarro & Gonçalves, 2020), which causes a cascade effect on an reduction of litter breakdown in the tropics. Thus, the conservation of riparian vegetation is important to keep the matter and energy inputs for the stream trophic chain.

Conclusion

Nutritional quality, i.e. palatability, presented a more important effect than the colonization time available to bacteria. For palatability, secondary compounds are less important than N, C and P. Regarding the conservation of Atlantic Forest streams, processes that degrade and homogenize riparian vegetation, reducing nutritional quality, can alter local decomposition. Processes such as urbanization and other land uses that degrade riparian vegetation alter the diversity and availability of nutrients for microorganisms, which influence ecosystem services and can reduce the abundance of decomposers, affecting the trophic web of these ecosystems.

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