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PROTOCOL
(Version 2)[1]

ESTIMATING DECOMPOSITION RATES AND LITTER STABILIZATION FACTOR USING TEA BAG INDEX (TBI)

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1. Introduction

Decomposition is an extremely important biological process for tropical forest ecosystems, especially with respect to nutrient cycling and carbon flow. In this context, the litter deposited on the forest floor is an important source of energy for these processes. Studies on litter decomposition have advanced our understanding of the effect of environmental conditions on decomposition rates. Investigations of this nature compose the information framework needed for modelling fluxes and carbon stocks derived from forest necromass - an important issue regarding the role of tropical ecosystems in the mitigation of global warming (IPCC 2003, 2006; Hiraishi *et al.* 2013).

Several methods can be used to estimate these decomposition rates, but the lack of standardization has caused difficulties for comparison between environments, complicating their application to global models. To try to overcome this difficulty, Keuskamp *et al.* (2013) suggested the Tea Bag Index method (see http://www.teatime4science.org) as an alternative for providing standardized data, as well as obtaining an integrated view of the decomposition process between different ecosystems. The basis of the TBI methodology is (i) the use of commercially available tea bags, that are (ii) easy to acquire and (iii) standardized by the same plant material and the same surrounding mesh. Generally, the tea bags are buried and after a certain time, the non-decomposed mass is measured, to give an estimation of both the decomposition rate and the litter stabilizing factor. This methodology can be easily reproduced due to the availability of, and access to, the commercial material and the low cost of implementing the experiment. Thus, it is possible to use this methodology equally in all of the PPBio research grids and modules, to generate a database with standardized information about the processes of decomposition in the most varied ecosystems. The TBI method allows for rapid field data acquisition and can be used, for example, as a reference for studies that seek to determine the influence of environmental conditions (e.g. soil, altitude, canopy opening, water table) on decomposition rates and stabilization factors at different regional scales. In addition, the results obtained can be used to compare flows and carbon stocks derived from dead organic matter between different ecosystems, allowing a better understanding of the role of each vegetation type in the global carbon cycle.

1.1 Concepts

In this protocol, decomposition is the degrading action on the most varied organic materials that compose the litter (e.g. leaves, twigs and small branches) deposited on the soil of terrestrial ecosystems (e.g., forests and savannas). The speed at which this process occurs is called **decomposition rate (k)**. Another important parameter defined here is **stabilization factor (S)**. This factor represents the pre-humification time of the organic matter, which is when the C:N ratio is low enough to indicate the end of the decomposition – it is the moment when the nutrients will be

available in the soil for the plants. The stabilization factor is an important parameter to better understand carbon sequestration processes, since that, after stabilization, organic matter tends to keep this carbon stored in the soil. The TBI method establishes the integration of these two parameters.

1.2 Objective

The objective of this document is to describe a general protocol for the application of the TBI method (Keuskamp *et al.* 2013) in the permanent plots of PPBio research grids and modules, to enable estimates of decomposition rates and litter stabilization factor for different ecosystems.

2. TBI Method: tea bag selection

The TBI method uses two distinct and standardized commercial teas from Lipton (Figure 1A): "green tea" (less recalcitrant) and "rooibos tea" (more recalcitrant). There are currently two types of tea bags available in the market: version 1.0 woven bags (nylon mesh) and version 2.0 non-woven bags (polypropylene mesh). Both have tetrahedral bags of edge length 5 cm and a 0.25 mm mesh. The two types have the same plant material in their interior, but different product codes (EAN - European Article Number): (i) "green tea" *Camellia sinensis* (Theaceae) 1.0 (EAN: 8722700055525) and 2.0 (8714100770542), which consist in the 89% of "green tea", and (ii) rooibos tea, 1.0 (EAN: 8722700188438) and 2.0 (8722700188438), which contains 93% A*spalathus linearis* (Leguminosae) stem tissues. Proper numerical identification of teas is important because it avoids the use of other types of teas that would not conform to the method. In addition, the final calculation of the decomposition rate (k) and litter stabilization factor (S) depends on the identification of the type of mesh that surrounds each tea bag. Therefore, the researcher responsible for the study should clearly specify which EAN was used to avoid uncertainties related to the different mesh types.

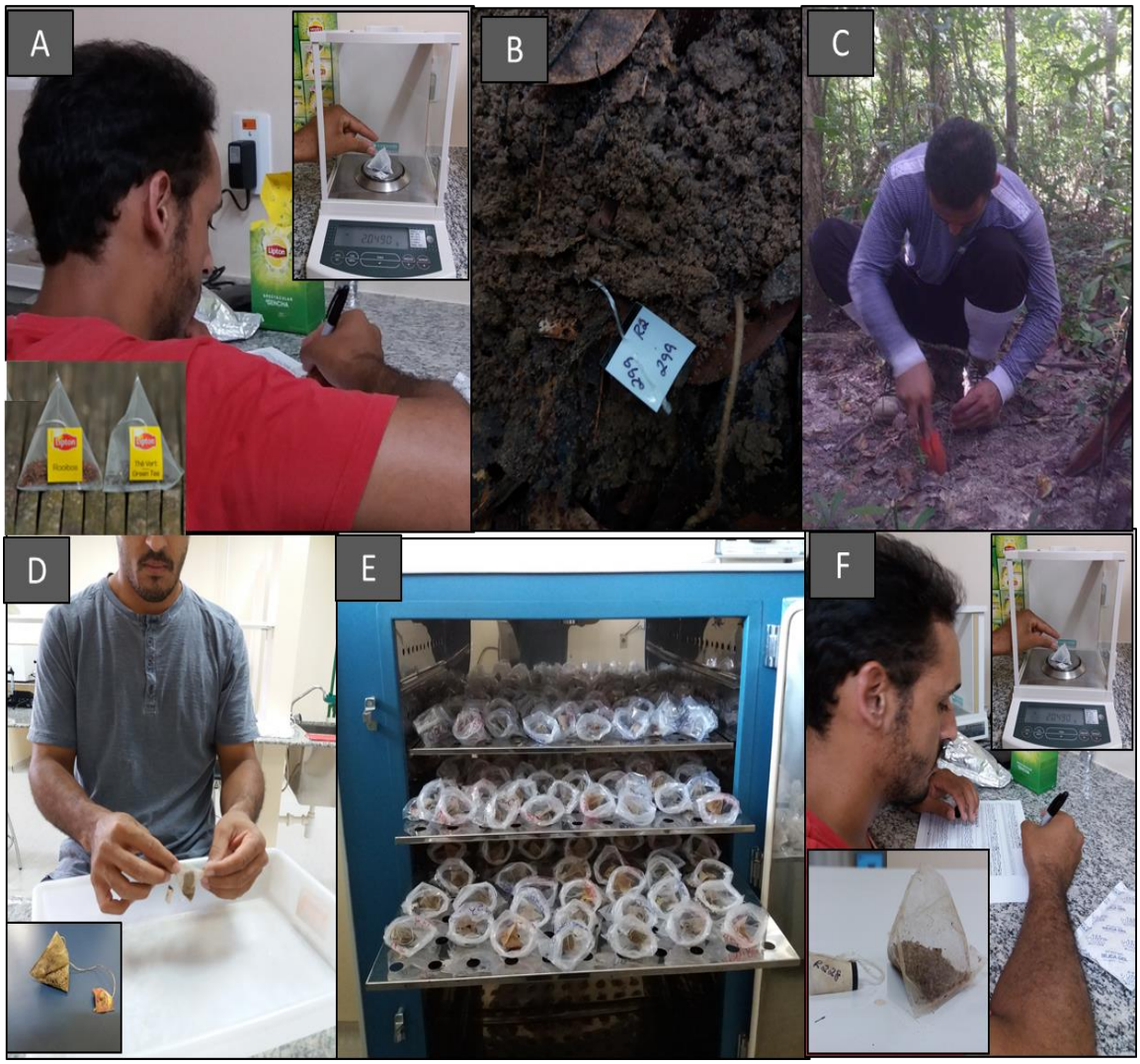

Figure 1 - Methodological procedures for implementation of TBI method: (A) weighing and identification of tea bags, (B) tea bags buried (incubated), (C) withdrawing tea bag, (D) triage of tea bags (physical integrity classification, according to item 9 of the protocol specified below), (E) tea bag drying and (F) tea bag weighing after drying.

3. TBI protocol adapted to PPBio modules and grids

The TBI protocol (Keuskamp *et al.* 2013) allows for the use of tea bags in any environment because of the simple installation rules. However, as a way of standardizing the installation of the experiment in the permanent plots of PPBio modules and grids, we are suggesting (i) the application of a larger number of tea bags per plot, (ii) the standardization of the position of the replicates in relation to the plot benchmarks and (iii) a shorter incubation time of the tea bags in the soil. These suggestions are derived from Silva´s 2018 study in the PPBio-Maracá Grid (Roraima) in 2017 (Silva, 2018).

The stepwise protocol:

1. Buy Green and Rooibos teas from Lipton in sufficient quantities for the number of plots to be sampled (check availability of teas at http://www.teatime4science.org/method/availability-of-tea/). Each Lipton tea box has 20 (or 25) units and we are proposing that at least six pairs (6 units of green tea and 6 rooibos) be installed in each plot. The installation of six pairs of tea bags (rather than just one pair) along the plot increases the chances of obtaining an average value for k and S rates per plot. This reduces the uncertainties related to a mean derived from only one pair of tea bags or if plots are left unmeasured due to losses because of severe fragmentation of the units by meso and macrofauna in the soil.
2. Before incubating tea bags in the plots, determine the initial weight (IW) of each unit using an electronic scale with an accuracy of 0.0001 g (0.001 g is also acceptable) (Figure 1A). This is important because weight variations (pre and post incubation) may be imperceptible in some cases.
3. Identify sample units with a black marker on the white side of the label attached to each teabag (Figure 1B).
4. Determine the best time of the year to install the experiment. According to Keuskamp *et al.* (2013) the best installation period is the "higher productivity" of the ecosystem. In the tests carried out in Roraima the tea bags were incubated at the end of the rainy season => at the beginning of the dry season. This period was considered the one with the greatest biological activity throughout the year in this region of the Amazon because the soil is still humid and the daily photo-period is longer than at other phases of the year (cf. Barbosa *et al.* 2012).
5. Next, bury the six pairs (six green units and six rooibos units) in each plot. The units should be buried at a depth of 8 cm, maintaining the following pattern: the first pair must be installed close to the first benchmark (0m), the second one at 50m and so on every 50m until the last benchmark at 250m[(2)]. The distance between the incubated units (green and rooibos) at each benchmark must be between 20-25 cm, keeping the labels visible aboveground (Figure 2A, 2B, 2C, 2D). Since the plots are permanent and should not undergo sudden environmental changes, the minimum amount of superficial litter should be removed and it must be replaced over the incubation site of the tea bags, in an attempt to guarantee micro-environmental conditions that characterize the soil surface in whole plot.
6. Mark the location with a coloured flag to facilitate the recovery, taking into account the dates, plot code, phytophisiognomy (optional) and any other observations concerning environmental conditions on the plot.
7. Remove tea bags after 42 days of incubation (6 weeks) (Figure 2D). The original method recommends that the collection of the bags should be up to 60 days post-

[2] **Increasing the number of pairs per plot may be advisable in places with intense meso and macrofauna activity in the soil. Thus, if financial resources are available, it is possible to incubate more pairs of green and rooibos tea in each benchmark.**

incubation for tropical regions. However, we are suggesting that this time must be reduced to 42 days. This is because our preliminary studies indicated that in the northern Amazonian conditions (high moisture and temperature) the ideal incubation time is set between 35-50 days. Thus, 42 days has been adopted as an average to prevent excessive tea bag losses.

8. After the collection, clean the outside of the tea bags by removing soil particles or roots adhered to the meshes. After cleaning, dry the tea bags for 48 h at 70°C in an accurate oven. Do not exceed this temperature, otherwise the mesh will be destroyed, and the dry weight of each unit will be lost.
9. After cleaning and drying, perform an evaluation of the physical integrity of each bag. Undamaged tea bags (N) are fully utilized for calculating k and S. Ripped tea bags (R) have lost material (total or partial) and are discarded because it´s impossible to use them in mathematical operations, but they can be used in calculations of the percentage of total mass loss (MacDonald *et al.* 2018; Seelen *et al.* 2019). Tea bags with small holes (H), generally caused by roots or mesofauna, can be used, but they need to be carefully evaluated by the lead researcher.
10. Remove the label from tea bags that will be used in the calculations, keeping the string attached because this is important for calculating k and S. Weigh each tea bag (FW = individual final dry weight).
11. The sequence of calculations to estimate the stabilizing factor (S) and decomposition rate (k) is defined by a series of mathematical formulas presented by Keuskamp *et al.* (2013). Excel spreadsheets with the sequence of calculations can be easily obtained in the Data submission for researchers NON-WOVEN BAGS and Data submission for researchers WOVEN BAGS (http://www.teatime4science.org/publications/).
12. Although the insertion of the individual IW and FW values of each benchmark can be applied to the spreadsheets, we are suggesting the use of an average of IW and FW of all replicates (green and rooibos teas) installed over each plot. This procedure is in accord to the PPBio sampling design (a plot = a sample) and strengthens the results because it uses all the tea bags without integrity problems, guaranteeing mean values of k and S for all plots investigated.

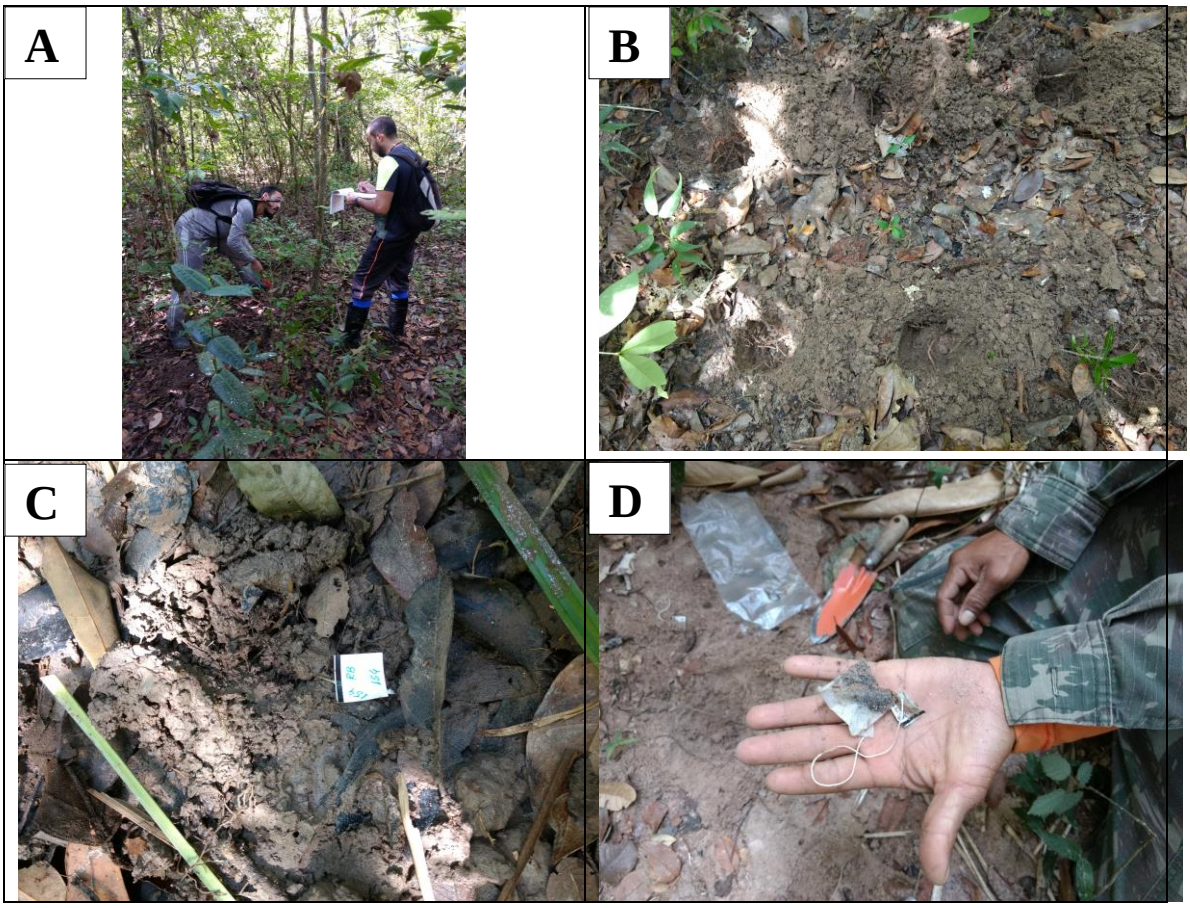

Figure 2 - Field procedures for installation and collection of tea bags: (A) Identification of the plots and benchmarks, (B) opening holes in the soil (8 cm in depth), (C) burial of tea bags keeping labels aboveground, (D) collecting tea bags (they must be preserved in plastic bags for further evaluation of physical integrity, cleaning of debris, drying and weighing).

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