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In-vitro Digestibility Organic Materials – Relation with Field Mass Loss Litter Bag Method

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ABSTRACT

Decomposition and litterfall are the primary mechanisms by which plants release their organic matter and nutrients into the soil, which helps prepare the stage for beneficial pathways in the restoration of damaged ecosystems. Species selection and allocation for the successful use of litter in ecological agricultural fields relies on knowing the mechanisms of plant litter decomposition and its influence on soil nutrients, which are crucial aspects of the ecosystem material cycle. In current study, *in-vitro* dry matter digestibility (IVDMD) used for evaluating quality animal feed reveals some potential in the decomposition of organic matter estimated. Nevertheless, some consensual advantages as laboratory incubation, this methodology demands a validation procedure. Therefore, the present work aimed to validate the IVDMD methodology by comparison with field buried litter bag mass loss, for 27 organic materials with different origins and chemical quality. The results reveal significant differences among the organic materials studied, reflecting their chemical quality variation, with digestibility values varying between 10.1 $g \cdot kg^{-1}$ in composted sewage sludge and 982.0 $g \cdot kg^{-1}$ in pig meat meal. IVDMD presented high accuracy results for all studied periods, with best results observed for 28 days incubation period ($r^2adj = 0.959^{***}$). Taking the chemical fractions that participated in initial decomposition process the IVDMD is a potential indicator of a labile decomposable pool of organic materials. Considering the high accuracy, repeatability (CV = 4.6%) and practicability, the IVDMD is a reliable alternative to the litter bag method in field mass loss availability.

Keywords: *in-vitro* digestibility, mass loss, litter-bag, decomposition.

INTRODUCTION

The carbon, nitrogen and nutrient cycle are regulated by plant species, resulting in providing herbivores and the decomposer subsystem with resources (Fig. 1) (Berg and McClaugherty, 2014). Several comparative studies have shown that functional features of live leaves impact litter quality and decomposition and continue until senescence (Pérez-Harguindeguy et al., 2000). Plant species that are frequently browsed tend to develop faster, have greater nitrogen concentrations in their tissues, and have lower quantities of secondary metabolites (Wardle et al., 2002). Based on the 'afterlife effect theory', higher-quality litter is produced by more appetizing species (Arisawa, 1998). This, in turn, increases decomposition rates by promoting the activity of the decomposer subsystem (Cornelissen et al., 1999). The decomposing remains of plants, including their limbs, leaves, flowers, fruits, and wilted roots, comprise what is known as litter (Berg, 2014; Hobbie, 2015; Tingyu et al., 2022). Climate, litter quality, and soil microbial and faunal communities are the three main factors that influence litter decomposition (Bradford et al., 2017;

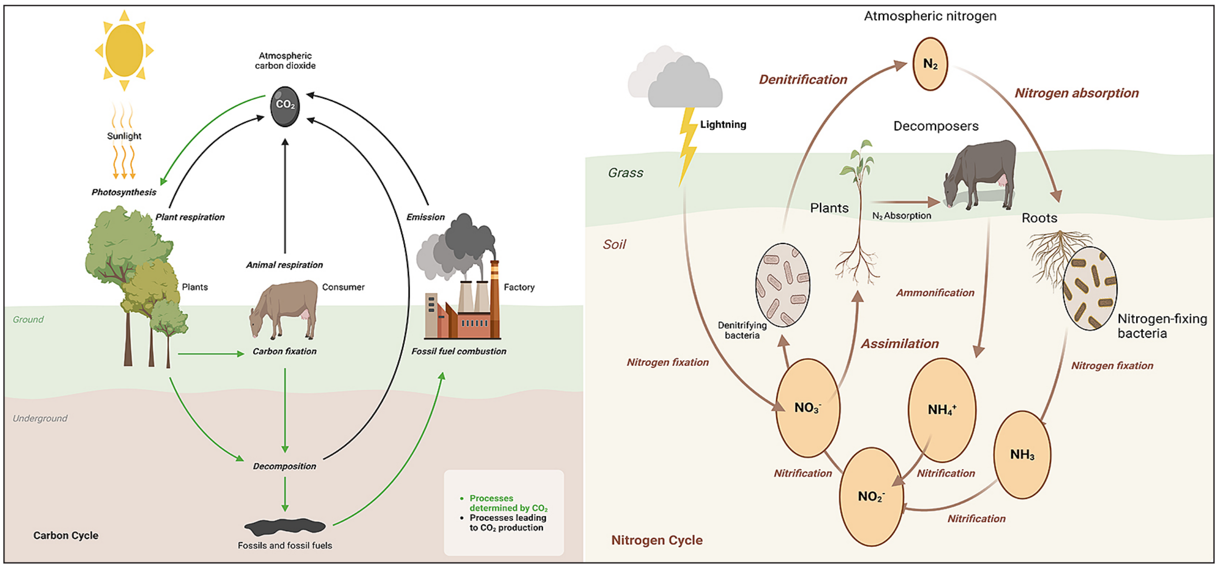

Figure 1. Carbon and nitrogen cycles that help in the decomposition of different matter

Couteaux et al., 1995; García-Palacios et al., 2013). Litter quality is a biotic component that significantly affects decomposition, but temperature and precipitation are essential abiotic drivers of decomposition in ecosystems (Fig. 2) (Parton et al., 2007). It is common for litter with higher nutritional content, fewer secondary chemicals, and less structural tissue or a bigger leaf area to degrade more rapidly (Wright and Welbourn, 2002). Furthermore, mass loss is often smaller in low-quality litter species compared to high- quality species (Almagro and Martínez-Mena, 2012; Brandt et al., 2007; Lee et al., 2012). This is because low-quality litter species typically have high C:N and lignin: N ratios.

The knowledge of decomposition process of organic materials applied to the soil is an essential tool to improving nutrient use efficiency. For the available methods in the study of the decomposition process, *in-situ* incubations are considered

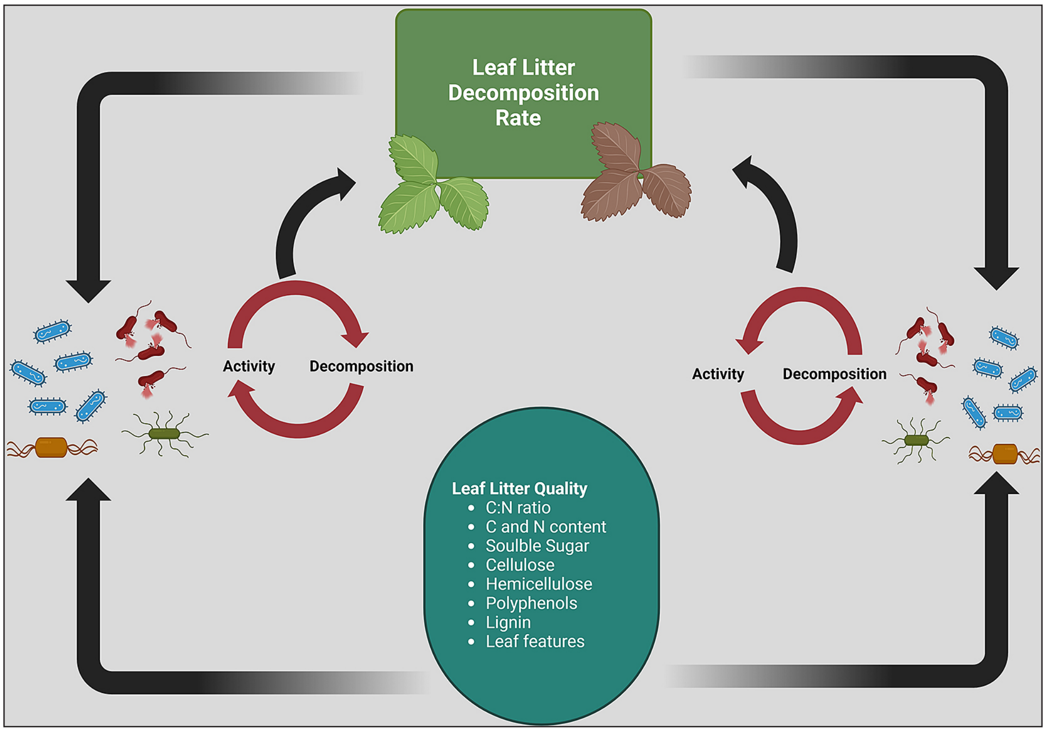

Figure 2. Different factors and key elements that participate in leaf litter decomposition

the most realistic technique, since they integrate the effects of temperature and humidity, which are the main influence factors in field conditions (Berg and McClaugherty, 2008). Between the various *in-situ* incubation methodologies available, the buried litter bag is one technique that reveals a greater emphasis, since their simplicity, low application costs and facility attainment results (Heim and Frey, 2004; Huang and Schoenau, 1997; Osono and Takeda, 2005). The higher capacity in the decomposition process analysis, translated by more accurate results seems to justify their use in these studies (Álvarez et al., 2008; Jalota et al., 2006; Vaieretti et al., 2005). Nevertheless, as field incubation experiment, the litter bag methodology was very laborious and time-consuming, which limited their practical applicability (Berg and Laskowski, 2006).

Currently, most of the existing methodologies to anticipate the decomposition process involve laboratory procedures which include soil incubations (Calderón et al., 2004; Kumar and Goh, 2003). The criteria for their use are obtaining a rapid and simple form to estimate the decomposable quantities under field conditions (Jarvis et al., 1996). However, developed in optimized conditions, the laboratory incubation is a potential estimation of the decomposition process, producing higher results to those obtained in field conditions with in-situ incubations, difficulty the interpretation and the extrapolation to field conditions (Herrmann and Witter, 2002; Reichstein et al., 2000; Wienhold et al., 2009).

Among the available laboratory incubation methods*, in-vitro* digestibility is used to evaluate the quality of animal feed (Apráez, 2020), reproducing the conditions existing in the rumen. In the original version, the rumen fluid and dry material mixture is placed in contact during a 24- to 48-hour incubation period (Clark and Mott, 2011). In an update of the original version, Tilley and Terry (1963) proposed a second stage related to the addition of an acidity solution of pepsin followed by a re-incubation for a new 48-hour incubation period under identical conditions. More recently, with the aim to reducing time and simplifying procedures analysis, other refinements have been proposed (Barnes et al., 1979). Most accepted and used, the Van Soest (1994) proposal replaced the pepsin acidified addition by washing the remaining residue from digestion with a neutral detergent solution. This modification maintains accuracy, improve the feasibility of the Tilley and Terry (1963) version and reveals high correlations with in-vivo version technique (Getachew et al., 1998).

Chemical composition of materials especially with more recalcitrant fractions are more sensitive to environmental factors such as temperature, moisture and microbial activity influence the predictability of IVDMD method, although the accuracy can be higher to allow the decomposition process to be evaluated.

Taking advantage of instrumental and analytical capacity installed in laboratories, the *in-vitro* digestibility method was used as an indicator of the potential decomposition of plants residues applied to soil. Mafongoya et al. (1997) suggests that the decomposition processes in the rumen and soil, even certain differences, are sufficiently similar to be used in comparative studies. Tian et al. (1996) supported this hypothesis, revealing that plant degradation during in-situ ruminant nylon bag assay correlated with decomposition in a litter bag study. Using plant residues, Cobo et al. (2002) and Tscherning et al. (2005) obtained highly significant correlations between the in-vitro digestibility technique and the litter bag decomposition method. Nevertheless, the few comparative studies with these methodologies have been performed only with plant residues (Cobo et al., 2002; Jensen et al., 2005; Shepherd et al., 2005). and little now with another type of organic residues from different sources and chemical quality. Therefore, this study aims to evaluate and compare the *in-vitro* digestibility of 27 organic materials from various origins and estimate the relationship with decomposition field results obtained with the buried litter-bag technique.

MATERIALS AND METHODS

Twenty-seven organic materials were selected taking the respective origin and chemical quality. A more detailed description of individual initial chemical characteristics and procedures used in their preparation and analysis was described by Sousa et al. (2016). The *in-vitro* digestibility, normally used to assess the dietary quality of animal food, was used as an indicator of the potential decomposition of the organic matter. For *in-vitro* digestibility evaluation, the methodology proposed by Tilley and Terry (1963), modified by Marten and Barnes et al. (1979). For an individual 50 mL Falcon tubes were weighed 0.25 g of dry material and added 7.5 mL of a buffered nutrient solution (Kansas State Buffer). A new 7.5 mL nutrient solution and 10 mL of inoculum rumen was added to each tube after 20 minutes the first addition. Two hours before the laboratory incubation, the rumen inoculum was collected in fistulated animals (sheep) and placed on a standardized diet. Thereafter, all Falcon tubes were closed and incubated at 39 °C under anaerobic conditions for a 48-hour period, shaking twice per day. At the end of the incubation period, 1.0 mL of 6N HCl and 5.0 mL of Pepsin (Difco certified) was added to stop digestion. This second phase was maintained for a 24-hour period at the same temperature and anaerobic conditions. Afterwards, the residue remaining was filtered, washed with hot distilled water and dried at 100 °C for a 24-hour period and the remaining organic material mass gravimetrically determinate. A dry sub-sample was taken for ash content determination, by heating at 550 °C for 2 hours (do Rosário G. Oliveira et al., 2000), to correct the weight of organic material remaining for contamination with soil. For the control treatment (blank), incubated with no organic material addition, was followed the same procedure. For each organic material and control treatment was realized three replicate samples to assess respective errors. The *in-vitro* dry matter digestibility (IVDMD) for each organic material were determined by using the expression relative to Equation 1.

$$IVDMD\ (g \cdot kg^{-1}) = (1 - W_d - W_b/W_s) \times 1000 \quad (1)$$

where: W_d was the weight of dry organic material remaining after the incubation period, W_b the weight of dry residues from blank, and W_s the dry weight of the original organic material sample, with *IVDMD* expressed in grams per kilogram of dry matter-free ash.

Results from *in-vitro* digestibility were analyzed by ANOVA, with statistical significance difference means determined by LSD test at 0.05 probability level. For the methodology validation precision analysis was evaluated based on repeatability criteria, calculating coefficient variation (CV), while accuracy analysis based in simple regression techniques established between IVDMD of the organic materials and the respective *in-situ* mass loss buried litter bag values (Sousa et al., 2016). Field mass loss litter bag and IVDMD regression relations were established to use this methodology to decompose field estimation. The relation was established for mass loss obtained for six sample dates during a period of 178 normalized days, which corresponded to baseline correction of temperature and soil moisture effects relative to 392 calendar days period of field decomposition (Sousa et al., 2016).

RESULTS AND DISCUSSION

In-vitro digestibility materials

The IVDMD values for the 27 organic materials under study are presented in Table 1. The results showed a highly significant ($p < 0.001$) effect of the organic type material, with variation values between 10.1 and 982.0 grams per kilogram of dry matter. A high variation results are observed

Table 1. Mean values results of *in-vitro* dry matter digestibility (IVDMD) ($g \cdot kg^{-1}$) for 27 organic materials under study

Organic material	IVDMD ($g \cdot kg^{-1}$)
Apple leaves	517.1
Chesnutt leaves	336.3
Coffee dregs	283.3
Commercial compost 1	156.6
Commercial compost 2	279.0
Commercial compost 3	294.8
Commercial compost 4	125.4
Corn stubbles	329.4
Cow faeces	156.2
Cow manure	168.7
Got manure	139.9
Grapevine leaves	464.3
Horse manure	262.6
Municipal solid waste 1	352.9
Municipal solid waste 2	570.7
Olive mill waste	195.4
Pig faeces	366.9
Pig meat meal	982.0
Poultry manure	659.1
Rabbit faeces	304.5
Compost sewage sludge	10.1
Sheep manure	225.4
Solid fraction dairy cattle manure	136.4
Vine grape marc	263.3
Vine grape stalk	250.9
Wheat straw	362.6
Lupinus luteus	640.3
$LSD_{0.05}$	106.4
Mean	372.2
CV (%)	4.6

result of the initial chemical quality organic materials selected (Sousa et al., 2016). For the organic materials studied, the most significant high digestibility value was registered for pig meat meal (PMM), with a loss of 98% of initial mass weight. High significant results are also observed in other materials like municipal solid waste 2 (MSW_2), poultry manure (PM), apple leaves (AL) and Lupinus luteus (LL), with loss values superior to 50% of their initial weight. The digestibility values observed in these materials are associated with highest labile pool dimension presented, supported by the significantly bigger water-soluble (bio)chemical parameter values comparatively to other materials under study (Sousa et al., 2016). The chemical quality, mostly of a protein nature, translated by a lower C:N ratio between 3.4 and 9.8 values, supported the significantly higher digestibility values registered in PMM and LL (Table 2). The major proportion of labile fractions facilitates accessibility and increases the energy balance associated (Corbeels 2001), enhancing microbial activity and, consequently, the material digestibility. Low digestibility values are observed in commercial composted 1 (CC_1) and 4 (CC_4), cow faeces (CF), goat manure (GM), solid fraction of dairy cattle manure (SFDCM) and composted sewage sludge (CSS) materials, not exceed 20% of their initial mass weight (Table 2). These materials present more high cellulose and lignin fractions content, that promote an increase of resistance to the decomposition process due to their recalcitrant nature (Osono and Takeda, 2005; Shepherd et al., 2005; Sousa et al., 2016; Zhang et al., 2008). This behaviour is especially noted in CSS material, where the low C bioavailability, expressed by high lignin content (323.2 $g \cdot kg^{-1}$), severely suppressed digestibility that does not exceed the 10% of the initial mass. Various authors, studying plant materials, confirm the repressive effect of structural components, observing a limitation of microbial activity and a decrease of digestibility with increasing of lignin content (Cobo et al., 2002; Setia et al., 2012; Smith et al., 1971; Tscherning et al., 2005). For the other organic materials studied, a more approximated behaviour, despite the significant differences registered (Table 2). In opposition to the materials previously discussed, these materials presented a more equilibrium between labile and recalcitrant pools, with a mean digestibility value of 30% of initial mass. For the commercial composted 2 (CC_2) and 3 (CC_3), horse (HM) and sheep (SM) manures, wheat straw (WS) and corn stubbles (CS) materials, the greater balance existent between Van Soest fractions of hemicellulose, cellulose and lignin allowed obtaining a intermediate results between PMM and CSS (Sousa et al., 2016). However, materials as vine grape marc (VGM) or vine grape stalk (VGS) presented a deviant behaviour independent of equilibrium Van Soest fractions observed (Table 2). The lower digestibility values in these materials are related with the presence of high concentrations of secondary compounds, in particular polyphenols (Sousa et al., 2016). As Kraus et al. (2004) and Olk et al. (2006) observed these types of compounds presented an inhibitory effect under microbial populations that participate in decomposition process, limiting the material's respective digestibility (Cobo et al., 2002). The results obtained reveal that IVDMD method can translate the effect of the quality of the organic material in decomposition process, with major and minor digestibility values observed in materials with highest labile and recalcitrant pools, respectively. These results suggest that IVDMD method, likewise aerobic biological incubations methodologies, are sensitive to important factors that influence the decomposition organic matter, namely chemical organic matter quality. This fact corroborated the ideas defended by Mafongoya et al. (1997), which justified comparative studies between *in-vitro* digestibility and other biological incubations, like litter bag decomposition, due to similar sensitivity aspects.

*In-vit*ro digestibility and *in-situ* mass loss relation

Field mass loss litter bag regression relations were established with IVDMD in order evaluate the potential of this last method to estimate field decomposition process. The regression results for IVDMD and the mass loss registered in each sample date are presented in Table 2. For all sampling dates studied the IVDMD are significant regressed ($p < 0.001$) with mass loss of the organic materials under field conditions, explain more than 73% of respective variation. The results observed are equivalents or superiors to others similar studies realized only with vegetable materials indicate that IVDMD method is a valuable field mass loss indicator, regardless origin or chemical characteristics materials (Cobo et

Table 2. Simple linear regression model estimated between *in-vitro* dry matter digestibility (IVDMD) ($g \cdot kg^{-1}$) and field mass loss (ML) ($g \cdot kg^{-1}$) date for 27 organic materials under studied

Time (days)	Regression model	r^2_{adj} #	S_{yx} &
24	ML_{24d} = 28.9 + 0.5844 * IVDMD	0.9303 ***	24.9
43	ML_{43d} = 51.41 + 0.6856 * IVDMD	0.8806 ***	26.9
56	ML_{56d} = 70.47 + 0.6998 * IVDMD	0.8656 ***	29.7
65	ML_{65d} = 85.45 + 0.7042 * IVDMD	0.8485 ***	31.9
109	ML_{109d} = 143.98 + 0.7395 * IVDMD	0.7671 ***	34.9
178	ML_{178d} = 256.14 + 0.7845 * IVDMD	0.7309 ***	37.3

Note: # – coefficient of determination adjusted; & – root square error; ***highly significant for a probability of 0.001%

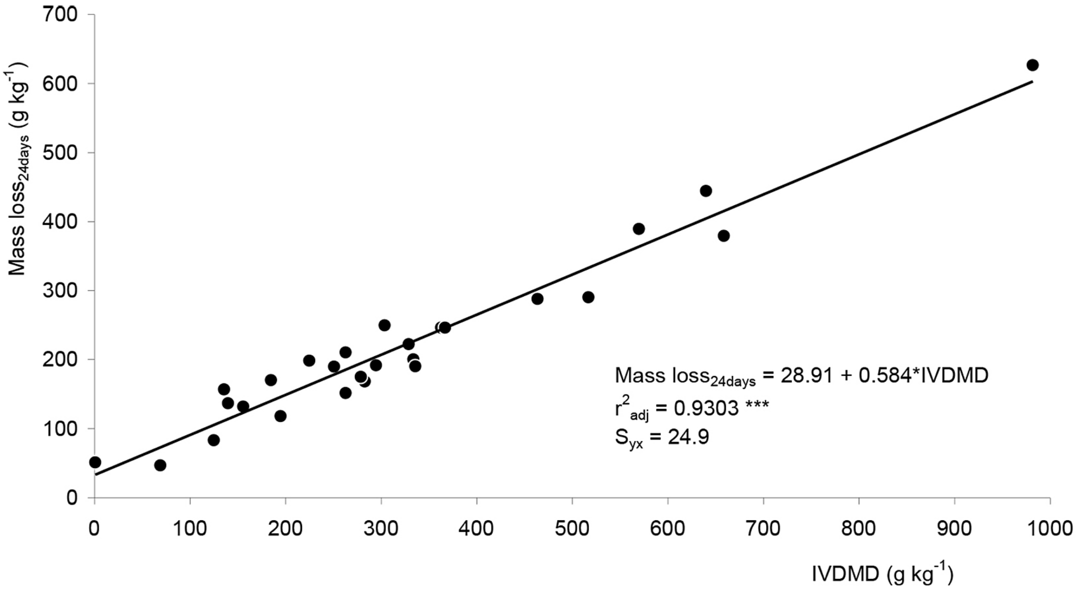

Figure 3. Simple model regression for mass loss (ML) ($g \cdot kg^{-1}$) after 24 incubation days and IVDMD ($g \cdot kg^{-1}$).

al., 2002; Tian et al., 1996; Tscherning et al., 2005). Nevertheless, for studied materials, the results also showed that IVDMD predictability accuracy varied with time incubation litter bag method (Table 2). The highest predictability results were observed for first 24 incubation days, where IVDMD explained proximally 93% of *in-situ* mass loss variation results (Fig. 3). With the increase of incubation time period was observed a decrease of predictability of IVDMD method, which explained only 73% of mass loss variation obtained in the longest incubation time of the litter bag method.

This predictability decreased tendency was related with implementation conditions of *in-vitro* digestibility methodology. Tipton et al. (2008) reported that constant temperature and anaerobic conditions applied in the digestibility method limit the size, diversity and activity of the microbial population. Vaieretti et al. (2005) mentioned another limited factor related to animal diet that can influence inoculum characteristics. These factors seem exert more limitation action in organic materials with high recalcitrant (lignin) or resistant (cellulose) compounds, where differences between IVDMS and *in-situ* mass loss are more significant (Fig. 4), or in more long incubation stages where these compounds are more predominant and influence (Cobo et al., 2002).

Beyond biological factors, the incapacity of IVDMD method to integrate temperature and moisture soil cycles effects can also help to explain the decreased predictability observed. Torres et al. (2005) referred the importance of these cycles through a physical time effect in materials structure, that promotes a partial lignin degradation and a reduction of protector effect on hemicelluloses and cellulose, contributing to the mass loss in field conditions.

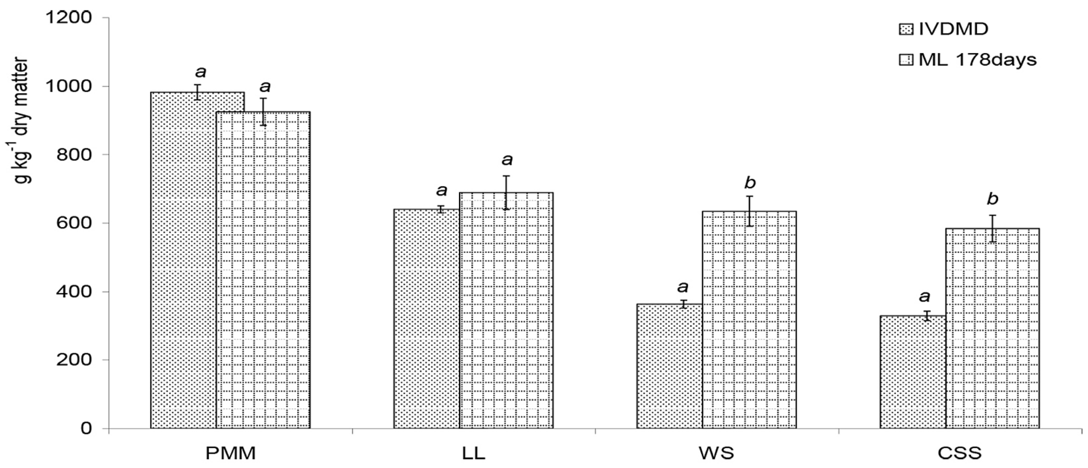

Figure 4. *In-situ* litter bag mass loss (g kg^{-1}) and *in-vitro* dry matter digestibility (IVDMD) (g·kg^{-1}) for materials with high labile (PMM, LL) and recalcitrant (WS, CSS) pools

The interception constant (b) value of the regression equation obtained for each sample date, possible reflects the importance of differences in conditions effects, which increase with time incubation and contribute for a major uncertainty of field mass loss estimation by IVDMD (Table 2). The highest correlation values obtained for 24 and 48 field incubation days reflect less influence of *in-vitro* digestibility method limitations conditions compared with long incubation times periods. Corbeels et al. (2000) associated these differences with the highest metabolic component compounds existing in the labile pool and the lowest activation energy associated with respective intervening enzymes (Stryer et al., 1995), promoting an easier decomposition independently of microbial population inoculums or limited medium conditions existed (Nahavandinejad et al., 2012).

Sousa et al. (2016) in regressed initial chemical characteristics of organic materials with field mass loss in various incubations time, observed major high significant correlations between labile pool indicators and initial periods. These results, together with high correlation quality results obtained between IVDMD and first-time field mass loss incubation (Table 2), indicated that soluble fractions are the main influence constituents of digestibility in organic materials. These results suggested that IVDMD is a potential indicator of the labile pool of organic materials. As a rapid incubation method, IVDMD can be used preferably in short-term studies to estimate the potential decomposition fraction of organic materials. In this context, the IVDMD method can be considered a reliable alternative to the litter bag decomposition field method, allowing a rapid, practically, reproducible (CV = 4.6%) and accurate assessment of mass loss field condition process. Therefore, digestibility can be incorporated in minimum data set defined by Laurance (1996), increasing the evaluation efficiency of the organic materials decomposition process. The findings of this study align with previous research in confirming the accuracy and practical advantages of in-vitro methods like IVDMD for estimating decomposition rates, particularly over shorter incubation periods. However, the study also introduces novel insights by emphasizing the significant variability in digestibility among different organic materials, which may impact the generalizability of in-vitro results across diverse environmental conditions. Similar to previous studies that have examined a range of organic materials, this study encompasses not only vegetable materials but also includes materials sourced from animals and urban or food industry waste. These by-products are commonly utilized to enhance soil fertility, underscoring the significance of evaluating their decomposition and nutrient release processes. Moreover, while acknowledging the limitations of in-vitro methods in replicating field conditions, the study underscores the importance of calibration and validation against in-situ data, especially for highly recalcitrant materials. Additionally, the study highlights a nuanced perspective on the reliability of long-term predictions, suggesting that while IVDMD demonstrates high accuracy for shorter periods, its effectiveness for extended temporal predictions may require further validation. Overall, these findings contribute to a deeper understanding of the conditions under which in-vitro methods can be reliably applied and the necessity for careful consideration of material-specific variability and environmental influences on decomposition dynamics.

CONCLUSIONS

Three main mechanisms contribute to the breakdown of plant litter: leaching, biotic processes, and abiotic processes. Because wetlands are unique ecosystems that include both land and water, the way their litter decomposes is slightly different from that of more conventional settings like woods, grasslands, and meadows. Soil organism metabolism, human activities, and environmental conditions all contribute to the entry of foreign compounds during decomposition, which alters soil structure, organic matter (SOM), and sedimentation, among other wetland performance metrics. Studies on the breakdown of plant debris are still in their early stages, particularly in these areas:

The breakdown of substances both above and below the earth. Most of the research has shown that above-ground litter decomposition occurs at a much faster rate than root litter decomposition and that there is a significant difference between both of them. However, the causes and consequences of this difference, as well as the relationship between root litter decomposition and soil physicochemical properties, are still poorly understood.

The availability of nitrogen and phosphorus determines the primary production capacity of terrestrial ecosystems. In vegetation, these elements are introduced by litterfall. There is little evidence of either effect on litter decomposition. Therefore, to come to a consensus, it is crucial to research the effects of N and P addition on litter decomposition both individually and in combination.

Changes in the sorption characteristics of soils in wetlands are caused by the breakdown of organic materials. The decomposition of organic materials in soil is accelerated by litter decomposition, which also alters the soil's physical and chemical characteristics and strengthens its structure. The discharge of industrial wastewater is a major source of heavy metal pollution in soils, and heavy metal adsorption in soil can reduce the mobility of these pollutants. The decomposition of litter may affect the capacity of the soil to retain heavy metals and other contaminants, although the exact mechanism is unknown.

Ecosystems in agriculture to different land fields have a variety of plant life, including understory and herbaceous plants in addition to tress species, and previous research focused mostly on litter breakdown by a single species. The decomposition of mixed litter and the impact of interspecific interaction on that process should thus now get primary attention.

Considering the application of IVDMD methodology to 27 organic materials population selected, the results obtained allow the following conclusions: (i) as laboratory methodology the IVDMD reveal high repeatability results, with CV mean results of 5%; (ii) the IVDMD is a good predictor of field mass loss, especially for initial stages of processes were reveal more highest accuracy results; and (iii) considering the elevated correlations obtained and the major influence of labile fractions for initial stages of decomposition process, IVDMD is a potential indicator of labile pool in organic materials. The results obtained showed that the IVDMD method is a reliable alternative to mass loss in-situ availability, allowed high precision and accuracy results in a rapid and practical form.

The IVDMD method may not be as reliable for highly recalcitrant materials, such as wood and bark, which contain high levels of lignin and other resistant compounds, whose decomposition process under field conditions is prolonged and related with activity of specific microorganism and whose methodology does not account for the effects of these factors that can influence the degradability of these compounds under field conditions, although predictions for periods longer than one year are still considered high (r2 = 0.709). To improve reliability, it is essential to calibrate and validate the IVDMD method specifically for these types of materials and consider using supplementary decomposition assessment methods to ensure accurate predictions.

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