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Multimodal assessment of flax dew retting and its functional impact on fibers and natural fiber composites

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Abstract
Flax (*Linum usitatissinum* L.) is an economically important fiber crop species as it produces long cellulosic fibers with high tensile strength. The first step in flax fiber extraction occurs after harvest via a process known as dew retting in which pulled (uprooted) plants are placed on the soil. During this process microorganisms from the soil and the phyllosphere develop on the plants, leading to partial decomposition of the stem tissues and facilitated mechanical extraction and improved fiber quality. Despite its importance, the management of dew retting is mainly based on empirical knowledge that makes the process difficult to control. In this study the dynamics of flax dew retting were investigated for the first time by combining targeted-metagenomics (metabarcoding), enzymatic activities and chemical and microscopic characterization of the stem. This multimodal approach indicated that intensive microbial colonization and major chemical changes in the stem cell wall composition occurred during the first weeks of retting leading to progressive fiber bundle decohesion in the outer stem tissues. The main changes could be explained by the degradation of pectin-rich and non-lignified thin walls of both the parenchyma cells surrounding fiber bundles, and the differentiating xylem and cambial cells. Field emission scanning electron microscopy suggested that the ultrastructure of the fiber secondary wall is weakly impacted by dew-retting. Analyses indicated that the mechanical properties of extracted technical fibers were improved during the last retting stages. Overall data on thermoplastic composites produced by a twin-screw extrusion and injection molding processes suggested that the retting degree had a small but significant effect on tensile properties.

Keywords. fibers, retting, metabarcoding, enzymes, tensile, composites

Abbreviations: CWR: Cell Wall Residue; FE-SEM: field emission scanning electron microscopy; OTU: Operational Taxonomic Unit; SEM: Scanning electron microscopy.

1 Introduction

The use of plant fibers is spreading to new applications that aim to replace synthetic fibers with environment-friendly natural fibers and prepare the predicted end of petrobased resources. Flax *(Linum usitatissinum)* is an economically important species as it produces long fibers (elementary bast fibers) that are distributed in bundles in the outer tissues of the stem (Bourmaud et al., 2018; Mussig and Stevens, 2010; Summerscales et al., 2010). It is these bundles that correspond to flax industrial or technical fibers and which are more or less dissociated depending on the process used in scutching and cleaning. Elementary bast fibers are single cells that show remarkable mechanical properties owing to their morphology and cell wall composition and architecture. Fibers have thick secondary walls that contain low levels of lignin (2-5%) and non-cellulosic polysaccharides (mostly galactans and glucomannans) (Day et al., 2005; Morvan et al., 2003; Rihouey et al., 2017). Fiber secondary cell walls also contain high amounts of crystalline cellulose content, with microfibrils that are oriented almost parallel to the main cell axis (Müller et al., 1998).

The first processing step in the extraction of fibers from the flax stem is retting (Tahir et al., 2011). In Europe, this is mainly done by dew-retting in which harvested flax is left on the soil leading to microorganism growth on the stem and partial degradation of cell wall pectins and hemicelluloses responsible for the cohesion of fiber bundles within the outer stem tissues, and between elementary fibers (Akin, 2013; Meijer et al., 1995; Requile et al; 2018; Sharma and Van Sumere, 1992). This process facilitates subsequent mechanical extraction of the fibers and improves their quality in terms of homogeneity and mechanical performances (Martin et al., 2013; Mussig and Stevens, 2010). The microbiology of bast fiber retting has long been of interest to the scientific community, and increasingly-sophisticated techniques have been used over the years to better understand the diversity of the main actors: bacteria and fungi. The first studies were based on culture-dependent methods (Henriksson et al., 1997; Rosemberg, 1965; Tanner, 1922), and from the 2000s, the use of molecular methods (e.g. Amplified Ribosomal DNA Restriction Analysis - ARDRA) (Tamburini et al., 2004), led to improvements in the identification of these microorganisms. More recently our knowledge of microbial diversity and community structure during retting has increased considerably thanks to the use of metabarcoding by High-Throughput Sequencing (HTS) (Djemiel et al., 2017; Zhao et al., 2016).

Since different industrial processes require a regular supply of uniform, high quality fibers, a detailed understanding of the ensemble of the different factors affecting fiber quality could provide indicators for improved management. However, controlling the variability of plant fibers is extremely complex as they are macromolecular biological structures whose architecture and composition is affected not only by genetic and environmental factors, but also by the processing steps, especially the retting step which combines both biological and abiotic mechanisms (Akin et al., 2001; Placet et al., 2018; Tahir et al., 2011). The development of a multidisciplinary approach allowing in-depth characterization of the effect of retting at different stages from harvesting to the product should lead to the upgrading of bioproduct developments such as biocomposites in addition to the classical textile use of flax fibers.

Despite the existence of multiple factors affecting fiber quality, there are only few data in the literature that have attempted to track the changes in plant stem structure, microbial diversity and cell wall degrading enzyme activity during field retting in relation with the final properties of technical fibers and composites (Liu et al., 2017a). In this study we have implemented a novel multimodal approach to investigate the dynamics of flax retting by combining targeted-metagenomics and analyses of enzymatic activity, together with chemical-, spectroscopic-, and microscopic-characterizations of flax straw collected in the field during the retting process. In order to see how the observed changes affected the nature of technical fibers, we then examine the morphology and properties of fibers collected at different retting stages. In fine the effect of retting on final products was examined by studying the properties of thermoplastic composites reinforced by fibers.

2 Material and methods

2.1 Plant materials and sampling. Flax plants (*Linum usitatissimum* L., Cultivar Lorea) were grown on a typical silt loam soil located close to CALIRA Martainneville in the north of France (49°99'60'' N and 1°71'43'' E). Flax was harvested in July 2015 (stage R0) and stems were retted for 56 days. The middle region (30 cm long × total swath height) of the flaw swath was collected every week (stages R1 to R8 corresponding to retting periods of 1 to 8 weeks respectively) during retting from five plots distributed on five swath lines (L1 –L5) in the retting field (**Supplementary Figure 1**) then stored at -20°C until analysis. Just after harvesting flax, some samples were also disposed in litter bags to evaluate dry matter loss during retting. Climatic data during the retting period was obtained from http://www.infoclimat.fr/observations-meteo/temps-reel/abbeville/07005.html (**Supplementary Figure S1**). Technical fibers were provided by CALIRA (Martaineville, France) after extraction by mechanical defibering of the flax straw collected at different stages of retting.

2.2 Targeted metagenomics. Genomic DNA (gDNA) of different stem samples was prepared and a composite sample constituted by pooling five replicas as described previously (Djemiel et al., 2017). Molecular markers were amplified respectively with the V3 - V4 regions of

the 16S rRNA primers for bacteria and the ITS2 primer for the fungi. Amplicons were generated and sequenced using Illumina MiSeq 2x250 bp technology on the GeT-PlaGe platform (GenoToul, INRA from Castanet-Tolosan) following the procedure described in Djemiel et al (2017) (Djemiel et al., 2017). Data were analyzed using different analysis pipelines; mothur (v.1.37.4) (Schloss et al., 2009) to treat 16S rRNA gene amplicons (Bacteria) and PIPITS (v.1.4.0) (Gweon et al., 2015) for ITS2 amplicons (Fungi). The clustering of sequences was fixed to 97% identity and taxonomic assignment of operational taxonomic units (OTUs) performed by BLASTs on NCBI when the taxonomic assignment did not provide information at the genus or species level with the dedicated databases (SILVA db for bacteria and UNITE db for Fungi). For statistical analysis (alpha-diversity), all samples are homogenized by performing a sub-sampling for the bacteria at 5 919 sequences and for the fungi at 86 050 sequences. Microbial DNA sequencing data sets supporting the results in this article are available at the EBI ENA with accession number PRJEB27872.

2.3 Enzyme activity measurements. Frozen stems (400 mg dry mass) were blended in 100 mL of 50-mM phosphate buffer (pH 7) for 1 min prior to filtration through a GF/A filter. The soluble fractions were used to perform the enzyme assays in triplicate on a microplate. Enzyme activities were determined at 25°C and pH 7, and were expressed as mmol or nmol g^{-1} dry bast tissue h^{-1}. Cellobiohydrolase, β-D-glucosidase, β-D-xylosidase, β-D-galactosidase and α-L-arabinosidase activities were assayed by measuring the release of the fluorogenic product 4-methylumbelliferone (4-MUB, Sigma-Aldrich, Germany) as previously described by Sauvadet et al (2016) (Sauvadet et al., 2016). Polygalacturonase activity was quantified in duplicate by measuring the reducing end groups formed during the enzymatic degradation of polygalacturonic acid (0.5 g L^{-1}) using 3,5-dinitrosalicylic acid (DNS) reagent and a galacturonic acid calibration curve as reported by Bleuze et al (2018) (Bleuze et al., 2018). After 1 h of incubation, the reaction was stopped by adding 500 µL of dinitrosalicylic acid. The solutions were then boiled in water for 15 min. The absorbance of the solution was measured at 510 nm using a microplate spectrophotometer (Versamax, Molecular Devices, USA). The phenol oxidase and peroxidase activities were determined using 5 mM L-3,4-dihydroxyphenylalanine as described previously (Sauvadet et al., 2016). For fluorometric assays, the deep-well plates were incubated in the dark for 3 h then the absorbance at 460 nm was measured using a spectrophotometer.

2.4 Microscopy. Five stems collected from the 5 plots were observed by scanning electron microscopy (SEM) with a tabletop scanning electron microscope (TM-1000, Hitachi, Japan) at an accelerating voltage of 15 kV. Both stem surface and hand–cut transverse sections (0.2-0.5 cm) were observed after air drying. Ultrastructure of the secondary walls was observed by high resolution field emission scanning electron microscopy (FE-SEM). Thin sections of the flax stems were dehydrated through a graded ethanol series, substituted with *t*-butyl alcohol and freeze dried. Sections were coated with platinum with an ion sputter coater (E-1045, Hitachi, Tokyo, Japan) and examined under FE-SEM (S-4800, Hitachi) at an accelerating voltage of 1.5 kV and a 2.5 mm working distance.

For immunohistochemical observations, small fragments (3 x 3 mm) of the stem portions were fixed in 70% ethanol then dehydrated using ethanol and acetone prior to impregnation and embedding in an epoxy resin (epoxy embedding medium, EEM hardener DDSA, and EEM hardener NMA; Fluka) (Chantreau et al., 2014). Immunolabelling of xyloglucan was performed using mouse IgG monoclonal (CCRC-M1, Carbosource) that recognizes the alpha-Fuc-(1,2)-beta-Gal glycan group of fucosylated xyloglucan. Immunolabelling was carried out on 0.5µm thick transverse sections as described previously using AlexaFluor 488 goat anti-rat IgG (H+L) (Life Technologies) as a secondary antibody. Sections were mounted in Eukit prior to observation by fluorescence microscopy (Nikon Eclipse TE300) (Chantreau et al., 2014).

2.5 Chemical analysis. All chemical analyses were performed on extractive-free cell wall residue (CWR) obtained from manually separated outer- and inner-stem tissues. CWR was obtained by extraction of flax tissues and of technical fibers using water followed by 80% ethanol (Crônier et al., 2005). Lignin and polysaccharide content of the cell walls were determined as described previously (Chantreau et al., 2014; Crônier et al., 2005) as i) acetyl bromide lignin by measuring absorbance at 280 nm (Johnson et al., 1961) and as ii) monosaccharide composition of polysaccharides using a two-step sulfuric acid hydrolysis (Seaman et al., 1954) followed by high-performance anion-exchange chromatography (HPAEC-PAD), respectively .

2.6 Colorimetry. The color of fiber bundles was measured on a set of 3 replicates for each retting stage using a colorimeter (CR-400, Konica Minolta, Japan). The results were expressed in the CIE L*a*b* color space system for the D65 illuminant with an observation angle of 10° and a 50mm² measuring area. The CIELAB color space decomposes the color into coordinates within a three-dimensional color space (L*, a*, and b*) as follows: L* is the lightness (black = 0 to white = 100), a* indicates redness/greenness, and b* indicates yellowness/blueness (McGuire, 1992).

2.7 Physical properties of technical fibers

Thermogravimetric analysis was performed on Hi-Res TGA 2950 (TA instruments Inc, USA) with a nitrogen

flow (40 ml.min-1) from 25°C to 1000°C and a temperature ramp of 10°C per minute. A differential thermogravimetric analysis (DTGA) was obtained based on the dry matter loss as a function of temperature. Before mechanical characterization, the fiber bundles were stored for a minimum of 48h in a climatic room at 23°C and 50% relative humidity. The fiber bundle samples were glued on 2 one-part plastic tabs with a UV-curing epoxy glue (DYMAX ultra light-weld®, DYMAX Europe GmbH, Wiesbaden, Germany) and with a gauge length of 10 millimeters. This gauge length was used according to previous work and should prevent a large contribution of the interface between fibers in the measurement of the mechanical properties (Bourmaud et al, 2017). The diameter of the bundle was then determined using an optical microscope Axio Scope.A1 equipped with an Axiocam (Carl Zeiss Microscopy GmbH, Jena, Germany). The diameter of the bundles was determined as the average of 6 values obtained by 2 measurements on 3 pictures taken along each fiber bundle. The cross sectional area was determined as described in standard NF T25-501 which considers circular approximation of the fiber l from the mean diameter (standard NF T25-501). Then, the tensile mechanical properties were assessed by Test 108.5kN machine (TestWell, Saint Ouen, France) equipped with a 500 N sensor at constant and controlled humidity and temperature of 23°C and 50% respectively. The crosshead displacement speed was 10 $mm.min^{-1}$. A strength-displacement curve was obtained from which the tensile stress, Young's modulus and strain of fiber bundles were determined.

2.8 Composites processing and characterization. The technical fiber bundles were chopped into a homogeneous batch with an average length of 3 mm by Fibres Recherche et Developpement (Troyes, France). Composites were produced using a laboratory scale twin screw extrusion (TSE) (Leistritz, Germany) using a homo-polypropylene (PP) to which was added an anhydride maleic grafted polypropylene (PPgMA) as described in Berzin et al (Berzin et al., 2017). Both polymer and fibers were introduced into the TSE by a gravimetric feeder (KCV-KT20 K-Tron, Niederlenz, Switzerland). For all experiments, the proportion of fiber-PP-PPgMA used was 20 / 77.5 / 2.5% in mass, respectively, the flow rate was 3 $kg.h^{-1}$ and the screw speed was 200 rpm. The composite strands were water-cooled in a cooling bath before being compounded then granulated in compounds having a length of approx. 5 mm. The compounds were introduced into a micro-injection molding machine (Babyplast 6/12 Standard) (Molteno, Italia) and directly dried inside the feeders. The specimens for tensile tests were injected at 205°C and 85 bar of pressure in a 2A type dumbbell-shaped mold (ISO 3167). The complete injection cycle of a specimen lasted 17 seconds.

The specimens were stored for a minimum of 48h at 23°C and 50% relative humidity according to ISO 291:2008 standard. Then, the specimens were tensile tested using a Test 108.5kN machine, fitted with a pneumatic side action grip (Instron France S.A.S) and a 2 kN sensor and an axial extensometer (Axial extensometer 3542, Epsilon tech, Jackson, USA). The crosshead speed was 1 $mm.min^{-1}$. A minimum of 8 specimens for each formulation were tested. From the strength-displacement curve, the Young's modulus and the tensile stress of composite material were determined.

2.9 Statistical analysis

A one-way analysis of variance (ANOVA) was performed to assess the effects of the degree of retting on the stem chemical composition, enzymatic activities, and physicochemical properties of the technical fibers and composites. ANOVAs were performed using SigmaPlot® software (version 12.0, Systat, USA). The Holm-Sidak method was used to perform pairwise multiple comparison procedures.

3 Results and discussion

3.1 Microbial diversity and biological activity

3.1.1 Evolution of microbial diversity

The evolution of the bacterial and fungal diversity on harvested flax plants during dew-retting was evaluated using targeted-metagenomics on 16S rDNA gene and ITS markers. The alpha-diversity metrics (**Supplementary Tables S1 and S2**) increase between R1 and R8 as evaluated by bacterial and fungal community richness (OTUs observed and Chao1 estimator), evenness (Heip's metric) and diversity (the Inverse Simpson index (1/D)). However, while we have overall more fungal than bacterial OTUs, all estimators are more important for the bacterial communities. Interestingly, the most important estimators of alpha-diversity **(Supplementary Tables 1 and 2**) were observed at the point R0 when the plants are uprooted potentially representing the contribution of the phyllosphere.

Investigation of taxonomic diversity (**Figure 1, Supplementary Tables 3 and 4**), showed that the majority of bacterial OTUs identified during retting were assigned to classes belonging to the Proteobacteria phylum (Gammaproteobacteria: 6 to 50%; Alphaproteobacteria: 14% to 22% and Betaproteobacteria: 8% to 18%), the Actinobacteria phylum (Actinobacteria: 5% to 13%) and the Bacteroidetes phylum (Sphingobacteriia: 4% to 15%) (**Figure 1A**). These classes were also identified as being the most abundant during our previous metabarcoding investigation of flax retting (Djemiel et al., 2017). In agreement with the high alpha diversity observed at R0 the different classes are distributed more homogeneously at this time point. Of note is the drastic reduction of Gammaproteobacteria that occurs during retting from R1

to R8 as reported previously (Djemiel et al., 2017). For the fungal OTUs (**Figure 1B**), the major classes present were the Dothideomycetes (34% to 47%) and Sordariomycetes (6% to 12%) belonging to the Ascomycota phylum, and the class Tremellomycetes (10% to 20%) belonging to the Basidiomycota in agreement with our previous study (Djemiel et al., 2017). In contrast to bacteria, the relative abundance of fungal OTUs at the class level changes only slightly during the latter stages of retting.

The observation that eight out of the ten most abundant bacterial OTUs (**Figure 1C**) were also among the top ten OTUs identified in a previous metabarcoding study on samples grown and retted in 2014 in the same geographical area but in a different field is interesting (Djemiel et al., 2017). Such a result could suggest a relatively high year-to-year similarity in the composition and dynamics of the retting populations between the two studies. However, differences in the relative ranking (e.g. Sphingomonas was ranked 1/10 in the previous study and 6/10 in the present study; Pantoea was ranked 5/10 in the previous study and 1/10 in the present study) would also indicate more subtle changes in microbial population dynamics.

Six of the top ten OTUs correspond to 4 genera (Sphingomonas, Massilia, Pedobacter and Rhizobium). These taxa have been associated with water-retting and were only identified, for the first time, in dew-retting in our previous work (Djemiel et al., 2017). The fact that these genera have now been identified in two independent metabarcoding studies not only provides further proof for their involvement in flax dew-retting, but also underlines the interest of the metagenomics approach. Metabarcoding also allowed the identification of an OTU corresponding to another genus (Chryseobacterium) also previously associated with water-retting thereby enlarging the collection of known bacterial taxa potentially involved in dew-retting. A more detailed examination of the evolution of the top 10 bacterial OTUs (**Figure 1C**) revealed important variations during retting. For example the abundance of OTU00002 (Pantoea) increases significantly from R0 to R1 before decreasing and finally disappearing at the end of retting. The observed profile is very similar to that observed in our previous study (Djemiel et al., 2017) and could be related to the known pectinolytic activity of the Pantoea genus (previously named Erwinia) on green flax fibers (Morvan et al., 1988). Other OTUs (e.g. OTU00001, Sphingomonas) show an inverse profile with a decrease from R0 to R1, followed by an increase throughout retting. *Sphingomonas olei* was recently isolated from oil-contaminated soil and biochemical characterization showed that it was unable to utilize L-arabinose and L-rhamnose, but could use D-glucose and D-mannose (Chaudhary & Kim, 2017). The bacteria is therefore more likely to be involved in metabolizing sugars potentially derived from more recalcitrant cell wall polymers such as hemicelluloses and cellulose that are generally broken down at later stages in retting (Akin et al., 1996). In contrast to the results obtained for bacterial OTUs, only half of the top ten fungal OTUs identified (**Figure 1D**) were also identified in our previous study (Djemiel et al., 2017)

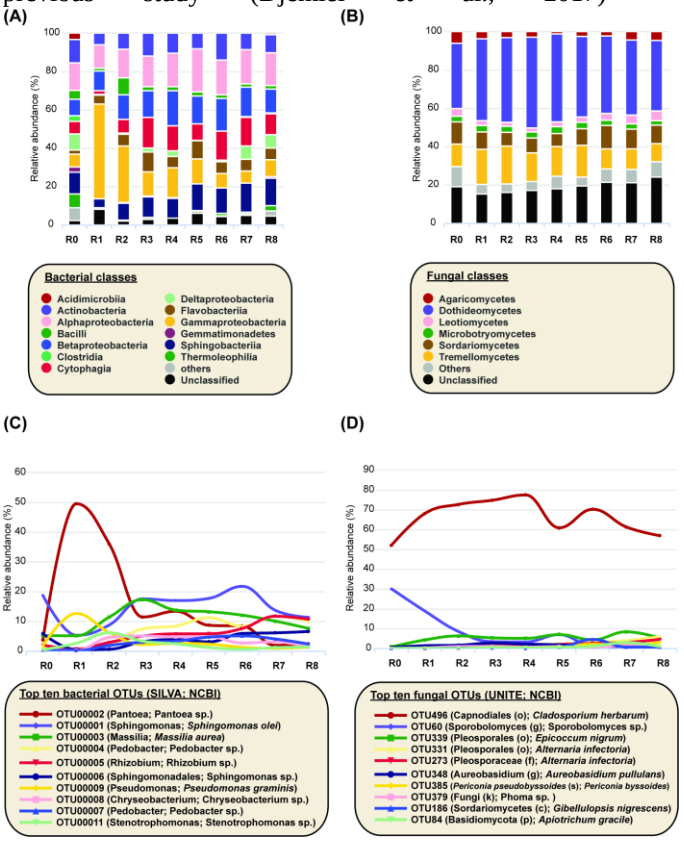

Figure 1: Relative OTUs abundance of bacterial (A) and fungal (B) at class level associated with flax straws during dew-retting. The consensus taxonomy for bacterial OTUs was assigned from the SILVA database and for fungal OTUs from the UNITE database. Relative abundance of the top 10 OTUs of bacteria (C) and fungi (D). The NCBI taxonomic assignment was performed by BLAST from the OTUs consensus sequences. Of the other five, one (Sporobolomyces) corresponds to a genus associated with decaying wood and forest litter degradation (Cadete et al., 2017) while another (Phoma) is involved in retting of different fiber plants (Brown and Sharma, 1984). The three remaining genera have not been previously associated with fiber plant retting but have been found on wheat and barley roots (Periconia (Dawson and Bateman, 2001)); on potato roots (Gibellulopsis (Lenc et al., 2012)) and are closely related to Trichosporum species known for their ability to degrade lingo-cellulose (Apiotrichum (Middelhoven et al., 2001)). Altogether these results could suggest that year-to-year variability of fungal retting populations is higher than that of bacteria, the ranking of the top five fungal OTUs ranking was more similar than that of bacteria. With regard to the evolution of fungal OTUs (**Figure 1D**), the most abundant by far is OTU496 (Cladosporium) representing between 50 and nearly 80%

relative abundance. The relative abundance of this OTU increases during retting up to R4 before decreasing to a value close to its initial relative abundance. Another relatively abundant OTU at R0, OTU60 (Sporobolomyces), decreases relatively rapidly reaching baseline levels at R3. Overall the targeted-metagenomic analyses indicate that microbial diversity for both bacteria and fungi increases during flax dew-retting.

3.1.2 Dynamic of enzymatic activities

Having established a detailed inventory of the microbial communities that develop during dew-retting we went on to determine hydrolytic and oxidase activities potentially produced by these microorganisms **(Figure 2).** Enzyme dynamics show that the highest activity of polygalacturonase (**Figure 2A**), phenol oxidase and peroxidase (**Figure 2C**) occurred just after uprooting flax (R0) then globally decreased until the end of retting. In contrast other polysaccharide-active enzymes (β-D-galactosidase, and β-D-xylosidase potentially acting on hemicelluloses and pectins) and cellulolytic enzymes (glucosidase and cellobiohydrolase) showed increasing activities for the first two weeks of retting before subsequently declining (**Figure 2B**) (Liu et al., 2017a). Albeit not significant, some enzyme activities tend to increase after swath turning (at R5). Overall enzyme activities are consistent with targeted-metagenomics showing the presence of a high microbial diversity with potential enzymatic functions during retting (Djemiel et al., 2017), as well as with previous studies reporting the presence of pectinases or hemicellulases associated with fiber retting (Liu et al., 2017a; Sharma and Van Sumere, 1992). The enzyme dynamics reported in the present study are consistent with the pattern of activities measured during hemp retting under controlled conditions where activities of enzymes active on polysaccharides increased during the first 2 weeks then declined to stage R4 (Bleuze et al., 2018).

Nevertheless, some care should be taken in associating enzyme activities measured during the initial retting stages specifically with microorganisms as it is possible that part of the overall activity might be due to the presence of biochemically active plant enzymes in the still living (but dying) up-rooted flax stems. Certainly, endogenous plant enzyme activity, including anti-oxidant peroxidases, is known to occur post-harvest in fruits and vegetables (Hodges et al., 2004).

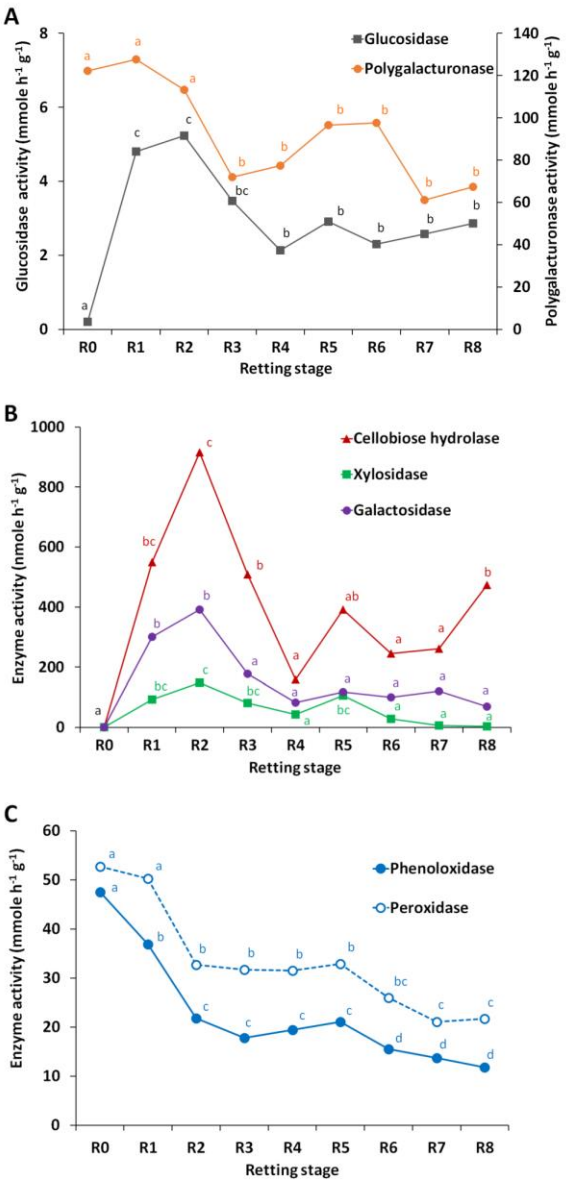

Figure 2: Enzymatic activities associated with flax straws during dew-retting A) polygalacturonase and glucosidase (mmol h^{-1} g^{-1} dry matter); B) cellobiose hydrolase, xylosidase and galactosidase (nmol h^{-1} g^{-1} dry matter); C) phenoloxidase and peroxidase (mmol h^{-1} g^{-1} dry matter)

3.2 Morphological and chemical changes of flax stems

3.2.1 Microscopic imaging of the stem tissues

Morphological changes to the stem essentially concern the outer tissues of the stem as previously shown (Akin et al., 1996; Tahir et al., 2011) (**Figure 3**). This observation is coherent with the apparent absence of any microbial colonization of stem inner tissues even during the later stages of retting (**Figure 3 A-F)**. In contrast, both fungi (**Figure 3B**) and bacteria could be observed at the stem surface after one week of retting (R1) and formed a mixed bacterial and fungal biofilm

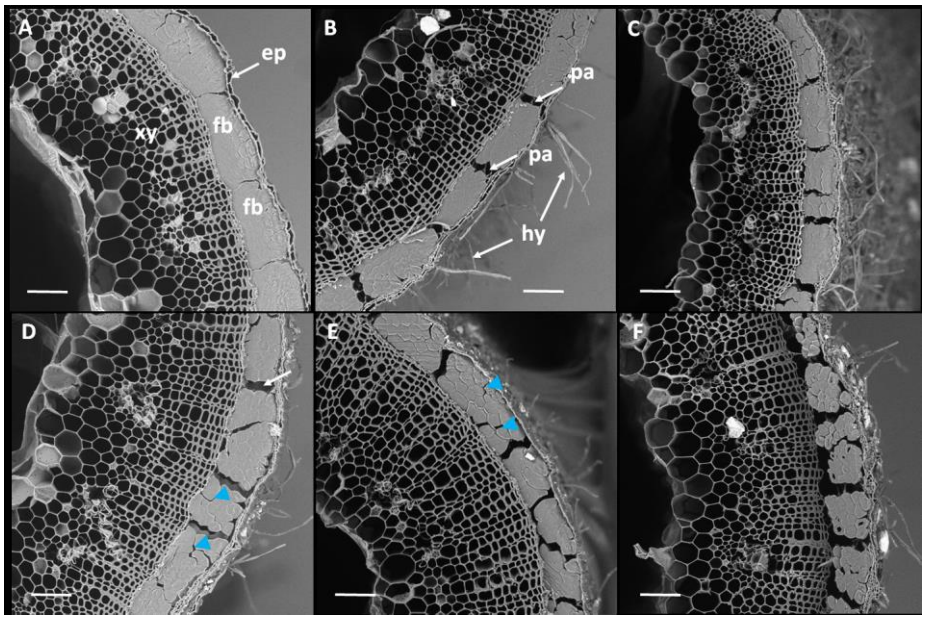

Figure 3: SEM imaging of transverse sections of flax stem at different retting stages: A) R0, B) R2, C) R4, D) R6, E) R7, F) R8; xy, xylem; fb, fiber bundles; pa, parenchyma, ep, epidermis; hy, hypha. White arrows and blue arrowheads indicate inter- and intra-bundle dissociation respectively. Scale bar = 100 µm

during later stages of retting (**Supplementary Figure S2**). While micrographs of stem transverse sections revealed colonization of the external (epidermal) cells of the bast-containing outer tissues during the later stages of retting (**Figure 3 B-F**), the observed changes mainly occurred to parenchyma cells located between the fiber bundles, in the compound middle lamella between fibers, and possibly in the epidermis (**Figure 3D**). Parenchyma cells between fiber bundles were easily distinguishable during early retting (**Figure 3B**) but were hard to observe at later stages (**Figure 3D-F**). Also separations between thick-walled fibers (arrowheads in **Figure 3D, E**) appeared during the later stages of retting. The dense microbial colonization of the epidermis makes it difficult to clearly evaluate the extent of any possible degradation of this layer. The cuticle which is rich in polyester and wax appears almost unaltered in agreement with the protective barrier role of this structure towards plant pathogens (Dominguez et al., 2017; Stimler et al., 2006). Nevertheless, it is known that phyllosphere and soil fungi can excrete cutinases that would facilitate microbial penetration into outer stem tissues (Aragon et al., 2017) where microorganisms would preferably use soluble components and non-recalcitrant polysaccharides as a carbon source. Overall, the microscopic changes in stem structure are in good agreement with previous observations during field retting (Akin, 2013; Akin et al., 1996).

In contrast to observed changes in parenchyma cells, SEM imaging did not reveal any visible modifications to the thick-walled fibers. This was further confirmed by FE-SEM allowing observation of secondary cell wall ultrastructure and fibrillary structures, most likely corresponding to cellulose macrofibrils (**Figure 4**). The microstructure of the secondary walls appears remarkably similar both before (**Figure 4A, B**) and after retting (**Figure 4C, D**), suggesting that the ultrastructure of the fiber secondary wall at the plant level (i.e. before mechanical extraction) is only weakly impacted by dew-retting. Although several studies have reported that retted fibers have higher cellulose crystallinity compared to unretted ones, the changes are relatively weak and are only observed during the last stages of retting (Mazian et al., 2018). Similarly, Bourmaud et al. (2019) reported that while retting impacts both the ultrastructure and the mechanical properties of scutched flax fibers due to increased cellulose crystallinity, the variations are slight and, once again, are only observed at the latter stages of retting.

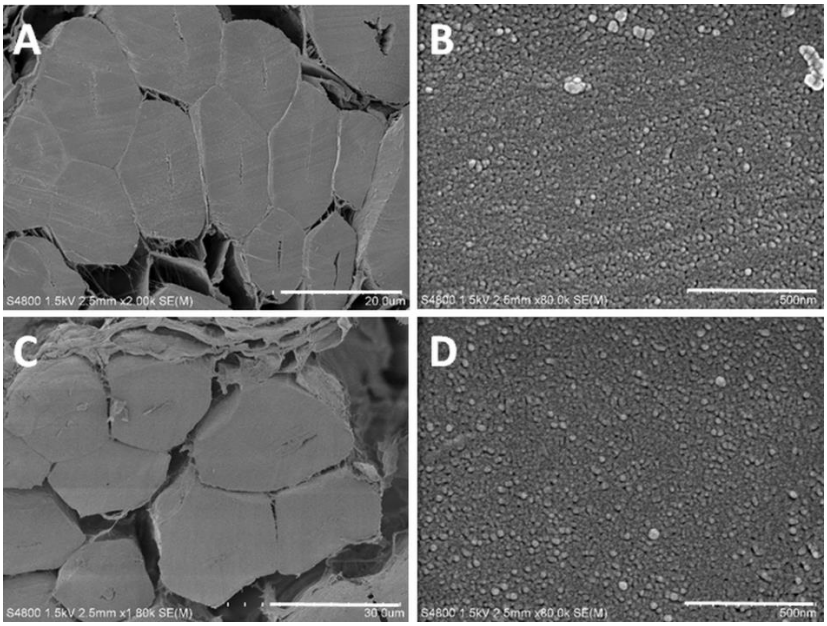

Figure 4: FESEM imaging of transverse sections of fiber bundles before (A,B) and after dew-retting (C, D). Scale bar: 20 µm (A, C); 500 nm (B, D)

Taken together, our microscopic observations suggest that field-retting facilitates the separation of stem tissues due to i) the dissociation of the outer bast tissues from the inner stem tissues and ii) the dissociation of fiber bundles from surrounding parenchyma (inter-dissociation) in agreement with literature data on dew-retting of fiber crops (Liu et al., 2017b; Tahir et al., 2011). As observed by Akin et al. (1996) the tissue architecture of the lignified xylem is globally unchanged reflecting the natural recalcitrance of lignocellulosic cell walls towards biological conversion (Himmel et al., 2007). The observed morphological changes suggest that microbial enzymes mainly target primary cell walls and the middle lamella of cells in tissues surrounding the inner core of the stem (i.e. cortex parenchyma and vascular cambium cells) . Notably the secondary walls are not degraded through the retting period even though microorganisms colonizing outer stem tissue can produce glycosyl hydrolases including cellulases (Djemiel et al., 2017). In addition to chemically-related cell wall polymer recalcitrance due to high cellulose crystallinity (Himmel et al., 2007) it is also possible that the propagation of microorganisms in the xylem may be hampered by fiber anatomy (long gap fibers with narrow lumen and sparse pits) (Wilson and Mertens, 1995).

3.2.2 Chemical changes of the stem tissues

SEM imaging is also consistent with our chemical analysis of outer tissues indicating that retting is associated with a rapid (within 2 weeks) decrease in the proportion of galacturonic acid and arabinose (**Figure 5A**) most likely reflecting the progressive degradation of middle lamella- and primary wall-pectin (Meijer et al., 1995; Morvan et al, 1988). In contrast glucose and mannose/galactose levels remain stable suggesting that cellulose, and secondary cell wall hemicelluloses and pectins are not degraded. These chemical changes are most likely related to degradation of the pectin-rich primary walls of parenchyma cells surrounding fiber bundles (inter-dissociation) and also the middle lamella component within fiber bundles (intra-dissociation) as observed in **Figure 4** and previously reported for flax field retting (Akin et al., 1996; Meijer et al., 1995; Sharma and Van Sumere, 1992). These parenchyma cells and cell walls represent a very low mass percentage of the outer tissues, thus leading to weak chemical variations. In this respect monomer analysis of outer tissues polysaccharides indicated that glucose is the main unit reflecting the high amount of cellulose. From a dynamic point of view, our results show that microorganisms colonize the stem surface and epidermis and produce extracellular enzymes active on cell-wall polysaccharides during the early stages of field retting (R1-R2). However, dissociation of fiber bundles occurs at latter stages (R4). Although such a lag probably reflects the time necessary for enzyme action to produce an observable change in tissue anatomy, it is also possible that abiotic factors could contribute to this process. Severe water loss has previously been proposed to contribute to stem tissue dissociation (Sharma and Van Sumere, 1992) it is therefore possible that the dry weather recorded between R4 and R6 (**Supplementary figure S1**) may also have an effect on the morphological changes observed in outer stem tissues.

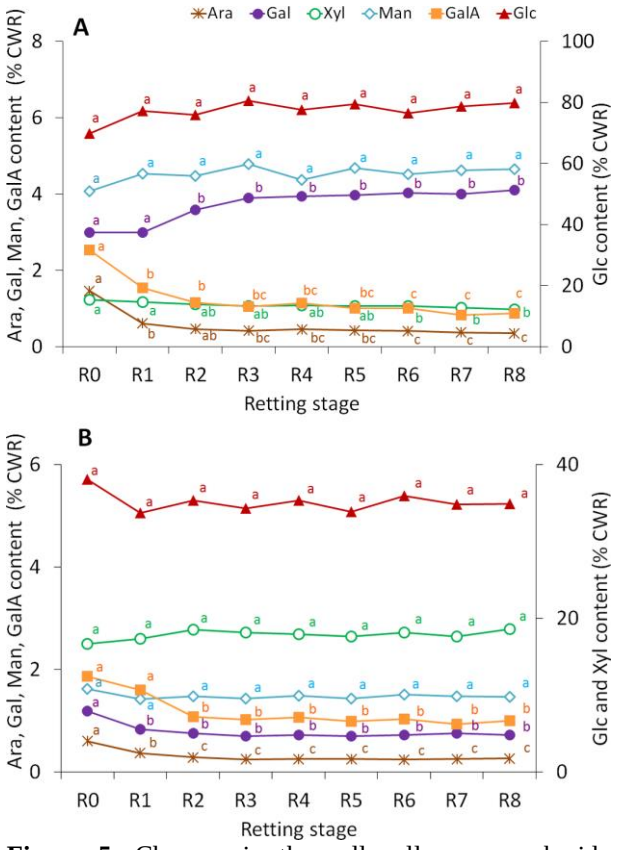

Figure 5: Changes in the cell-wall monosaccharide composition of the outer (A) and inner (B) stem tissues during dew-retting

Most studies on the impact of retting on the chemical composition of flax stems have concentrated on the bast-containing outer tissues and have generally ignored the inner stem tissues (Akin et al., 1996). Nevertheless, to complete the picture of how retting affects the flax stem we also analyzed the inner tissues. As observed for outer tissues, the sugar analyses show a significant and rapid (within 2 weeks) decrease in the proportion of galacturonic acid, galactose and arabinose levels. These changes are most likely related to the degradation of pectin- /hemicellulose-rich cell walls. In contrast, low change in glucose and mannose /xylose levels suggested little degradation of cellulose and secondary-wall hemicelluloses (**Figure 5B)**. The observed chemical alterations during the first two weeks are therefore potentially explained by the degradation of pectin-rich and non-lignified thin walls of cambium cells and differentiating xylem cells close to the cambium. Accordingly the lignin content of xylem CWR slightly increased from 27.0% to 30.3% after two weeks retting then remained stable during the latter stages of retting (data not shown). Overall the chemical data are in accordance with SEM observations of xylem that showed no visible microbial colonization nor morphological alteration/cell individualization in xylem during early retting. In contrast, cell wall immunolocalization of xyloglucan antibodies indicated that the cambium layer and the outermost layer of differentiating xylem disappeared after 2 weeks (**Figure 6**). These results thus provide new insights into the effect of retting on local changes of inner tissues of flax.

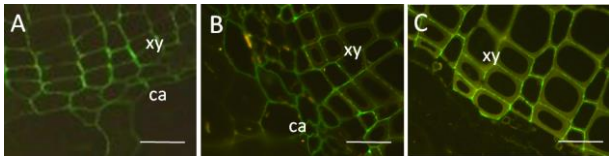

Figure 6: Fluorescence microscopy imaging of xyloglucan immunolabelling of the stem at different retting stages A) R0, B) R1, C) R2. xy; xylem, ca, cambium. Scale bar = 20 µm

3.3 Characteristics of technical fibers and derived composites

3.3.1 Retting impacts the physico-chemical properties of the technical fibers

Colorimetry is a rapid and nondestructive method that provides an objective means for color determination. This technique can therefore be used to characterize and classify plant fibers for industrial purposes (Akin, 2010). The CieLab color space system determines the coordinates of the colour vector in a colour space a*, b* and L*. The color measurements of the fibers extracted at R4 to R8 stages of retting (**Figure 7**) indicate a significant increase (10 to 23%) of lightness (L) between R4 and the last stages of retting. The opposite was found for the red (a) and the yellow (b) components that decline by 42 and 59% at the R7 and R8 stages, respectively. Overall color measurements correspond to the transition from yellow-grey to dark-grey color of the flax fibers during the last stages of retting. These changes might be explained by the presence of remaining microorganisms at the fiber surface. The CieLab values are in good agreement with values published earlier for field retted flax fibers (Akin et al., 2000; Martin et al., 2013).

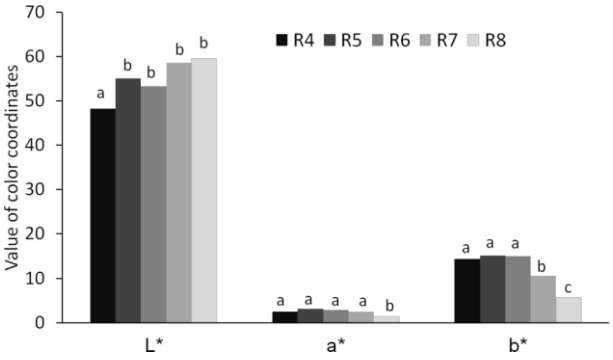

Figure 7: Changes in the CIELAB color values of technical fibers extracted at different retting stages. L*(lightness), a* (redness/greenness) and b* (yellowness/blueness)

Table 1: Effect of retting stages on the thermal properties of flax technical fibres

	peak 1		peak 2		peak 3	
	Temperature °C	Weight loss %	Temperature °C	Weight loss %	Temperature °C	weight loss %
R4	256.4	8.0	355.4	61.6	481.3	21.5
R5	249.3	6.9	354.2	62.3	477.3	22.1
R6	262.4	8.1	359.9	62.5	494.5	20.8
R7	270.5	6.7	367.3	67.8	493.4	17.8
R8	265.4	7.1	365.3	65.4	501.5	18.4

Concerning the thermal properties of technical fibers, the thermograph profiles indicate a slight increase in the thermal stability of the technical fibers between R4 and R5 retting stages and the last stages (R6, R7, R8) (**Supplementary Figure S3**). All samples displayed an initial weight loss at 40-50 °C which corresponds to absorbed water. The thermal characteristics associated to the degradation of the fiber carbohydrates were determined from the thermal derivative curve as weight loss percentages and corresponding peak temperature (Table 1). The peak corresponding to more labile components (hemicellulose and pectin) (Mazian et al., 2018) increased from 256 °C (R4) to 265 °C (R8) and was associated with a slight reduction in weight loss in this temperature range. Accordingly chemical analysis of the technical fibers indicates a very weak decrease in non-cellulosic polysaccharides containing Ara and Xyl (Fig. 8) in agreement with observed values for outer tissues. In contrast thermographs showed higher weight loss at temperature (peak 2) close to 350 °C. This second peak, which shifted towards a higher temperature from R4 to R8 is attributed to the thermal degradation of cellulose (Barneto et al., 2011). Accordingly this temperature represented the main contribution (61–65 %) in the weight loss of the flax fiber that contains high amounts of cellulose (Fig. 8). Removal of pectin-hemicellulose components during retting may partially account for the observed higher cellulose crystallinity in retted fibers (Bourmaud et al., 2019), and may also explain the shift in thermal decomposition that occurs with retting stage as reported for native fibers (Mazian et al., 2018; Sharma et al., 1999; Sisti et al., 2016). The thermal degradation at higher temperature (480 °C–500 °C) corresponds to around 20 % weight loss. This peak could be assigned to different components such as lignin and cutin remaining at the surface of the fibers (Manrich et al., 2017; Moriana et al., 2014). However considering the very low proportion of these components, notably lignin (< 2 %), the rather high weight loss occurring at 400 °C–500 °C could also be related to the volatilization of char (Barneto et al., 2010).

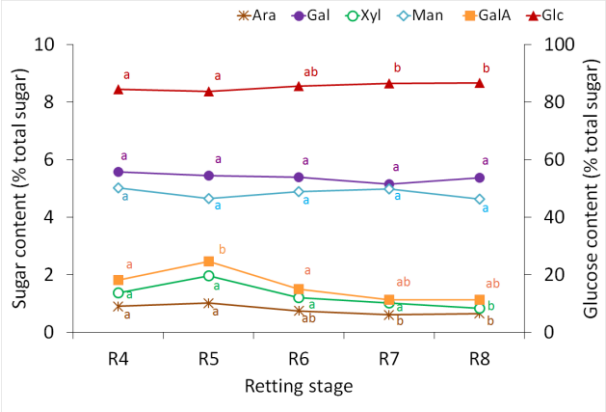

Figure 8: Effect of retting stages on the monosaccharide composition of flax technical fibers

Tensile tests were then performed on fiber bundles obtained from three different stages of retting (R4, R6 and R8) in order to evaluate the effect of retting on the mechanical properties (**Figure 9 A,B**). Our results showed that the Young's modulus and tensile strength were higher at R6 and R8 compared to R4. The lower fiber bundle strength observed at R4 could be related to the larger diameter of the R4 fiber bundles compared to R6 and R8 (**Figure 9 C**). Indeed, the tensile strength of plant fibers bundles is dependent on their diameter (Haag and Mussig, 2016; Mussig and Stevens, 2010; Placet et al., 2012; Sharma et al., 1999). The overall tensile properties of R6 and R8 technical fibers were comparable, albeit slightly higher for R8. This result is consistent with the absence of any significant variation in the content of cellulose-related monosaccharide (glucose) that represents almost 90% of total fiber polysaccharides at the R6 and R8 retting stages. It also suggests that R8 is not over-retted. Our results are comparable to the range of values reported in the literature on flax fiber bundles albeit towards the lower end. This may be related to the fact that mechanical tests

were performed on rather larger bundles (diameter > 100µm) than reported elsewhere, thereby explaining the weaker tensile properties. Variability in obtained values can also be due to the measurement technique utilized. Recent work have reported on the importance of calculating the section area and elliptic approximation would allow a more efficient and reliable assessment of the cross-sectional area of the fiber bundle (Haag and Mussig, 2016; Garat et al al, 2018). However such measurements need facilities enabling quasi 2D measurements as laser based fiber dimensional analysis system. As observed by Haag and Mussig (2016), measurements of tensile strength values can range from 470 -1465 MPa depending upon the method used to measure the geometry of the bundles. However these studies differ in the value of the correction factor to convert spherical estimation into elliptic estimation of the section area, and this impact differently on the calculation of the mechanical properties. Overall, our mechanical analysis is in good agreement with a recent study on hemp showing that i) field retting has a significant impact on the tensile properties of fiber bundles during the early stages, and ii) that longer retting does not significantly impact tensile bundle properties (Mazian et al., 2018). These results are also in agreement with those of Charlet et al. (2010) who reported only weak variations between the tensile properties of single flax fibers measured at different late retting stages. However, although longer retting does not appear to have a significant effect, over-retting can sharply decrease the tensile properties of hemp fiber bundles (Placet et al., 2017).

3.2.2 Retting has a moderate but real impact of the tensile properties of thermoplastic composites

As expected, extruding PPgMA with flax fibers generated fiber composites with improved mechanical performances compared to the matrix polymer alone (**Figure 10**). The natural fiber composite samples tested showed a slight but significant increase in the Young's modulus value from the stage R6 onwards. In contrast, the observed tensile strength values did not show a clear pattern and although they increased from R5, they generally showed a more erratic evolution. Dissociation of the fiber bundles will be facilitated by retting while improving the mechanical properties of the technical fibers. Thanks to this dissociation and further processing (extrusion) fiber would have cleaner surface which mat benefit to the interface between the polymer matric and the fibers. In addition although mechanical properties of the technical fibers get improved after retting, extrusion and injection can also impact on the fiber (morphology and mechanical properties. As a matter of fact, individualization of the fibers would lead to a better dispersion of the composites with positive effect on the tensile properties considering fibers at the late retting stages. Interestingly, a similar trend was very recently obtained for hemp composites (Mazian et al, 2020). While the performances measured in our

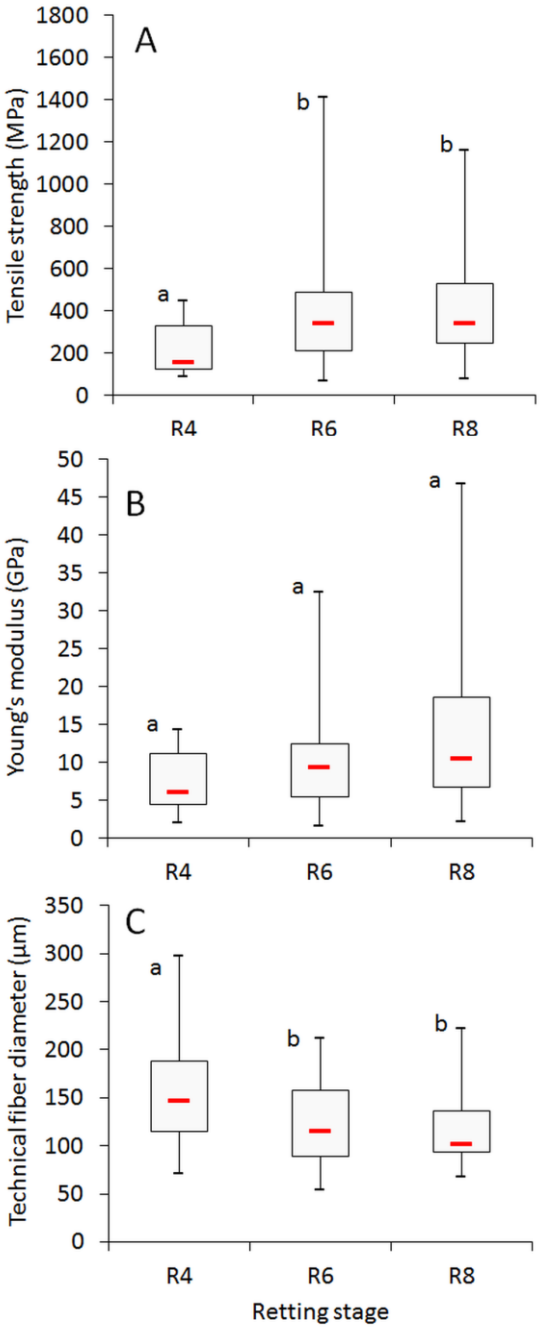

Figure 9: Effect of retting stages on the mechanical properties of the fiber bundles. Tensile strength (A), Young's modulus (B), diameter of fiber bundles (C)

study were lower than those observed in a previous investigation into the effect of retting on flax composite tensile properties this may be due to the severity of the compounding process as shown previously (Haag et al., 2017; Martin et al., 2013). Another key factor affecting mechanical properties of natural fiber composites is fiber morphology that is itself controlled by twin screw extrusion (Berzin et al., 2017). Our analyses showed that

the morphology of the short fibers was drastically modified after the whole process (compounding and

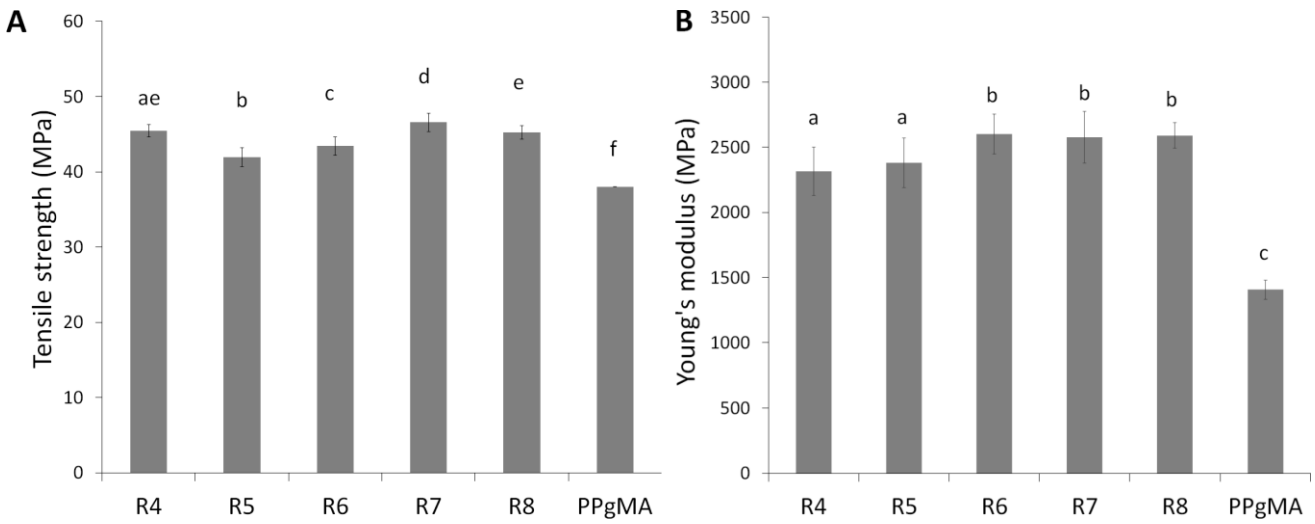

Figure 10: Effect of retting stages on the mechanical properties on the mechanical properties of the NFC PPgMA-composites. Tensile strength (A); Young's modulus (B)

injection) with the ratio length/diameter decreasing from 39-49 for the raw fibers to 7-8 after processing (data not shown). Although these ratios are lower than those reported in the previous work of Martin et al. (2013), this may be due to their use of a single screw extruder working at 20 rpm compared to a twin screw extruder at 200 rpm (this study) (Haag et al., 2017). The intrinsic properties of the reinforcement fiber elements (mechanical properties and morphometry) are also known to modulate the natural fiber composite microstructure thereby affecting the homogenized tensile properties of fiber composites (Tanguy et al., 2016). It is possible that the degree of retting may contribute to variations in microstructure (core/skin effect) that could explain the erratic trend observed in tensile strength values (**Figure 10**). Altogether, our results show that composite mechanical properties are improved by retting.

Insert Figure 10

4. Conclusion

Dew retting of flax is a complex dynamic biological process that involves progressive and subtle changes in stem structure and cell wall composition leading to facilitated fiber extraction and improved quality. The combination of different techniques in a multimodal approach has shed light for the first time on how different factors contribute to these changes, thereby opening the way to improved understanding and better management of dew retting that should contribute to reducing the variability of industrial flax fibers and improving composite quality.

Conflict of Interest

The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

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Supplementary material

Figure S1

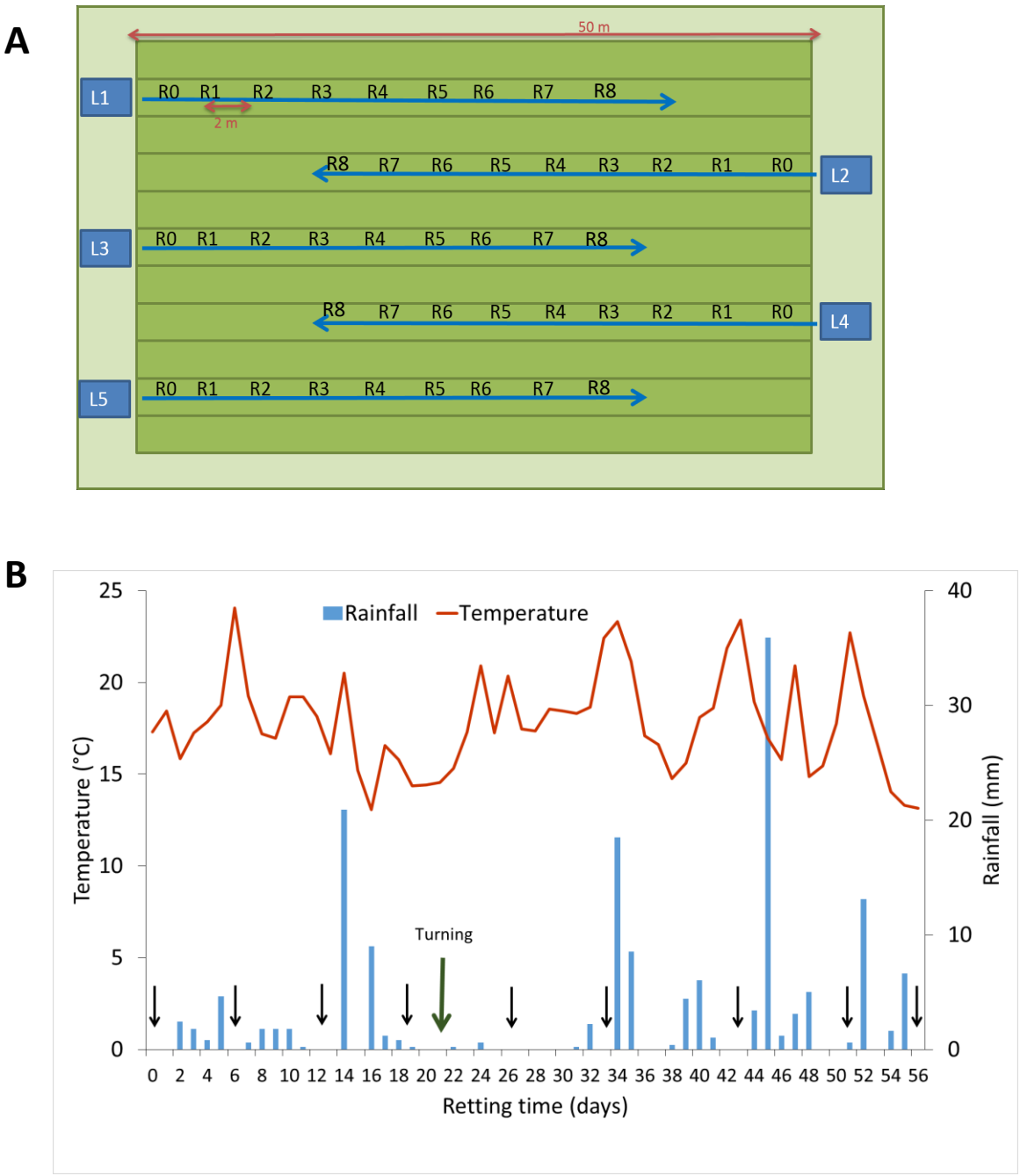

Figure S1 : Experimental conditions of field retting: A) experimental plots B) climatic conditions. Samplings are labeled as R0 to R8 on each of the 5 replicates at regular intervals (see B, black arrows). The swath were turned once during retting (see B, green arrow)

Figure S2

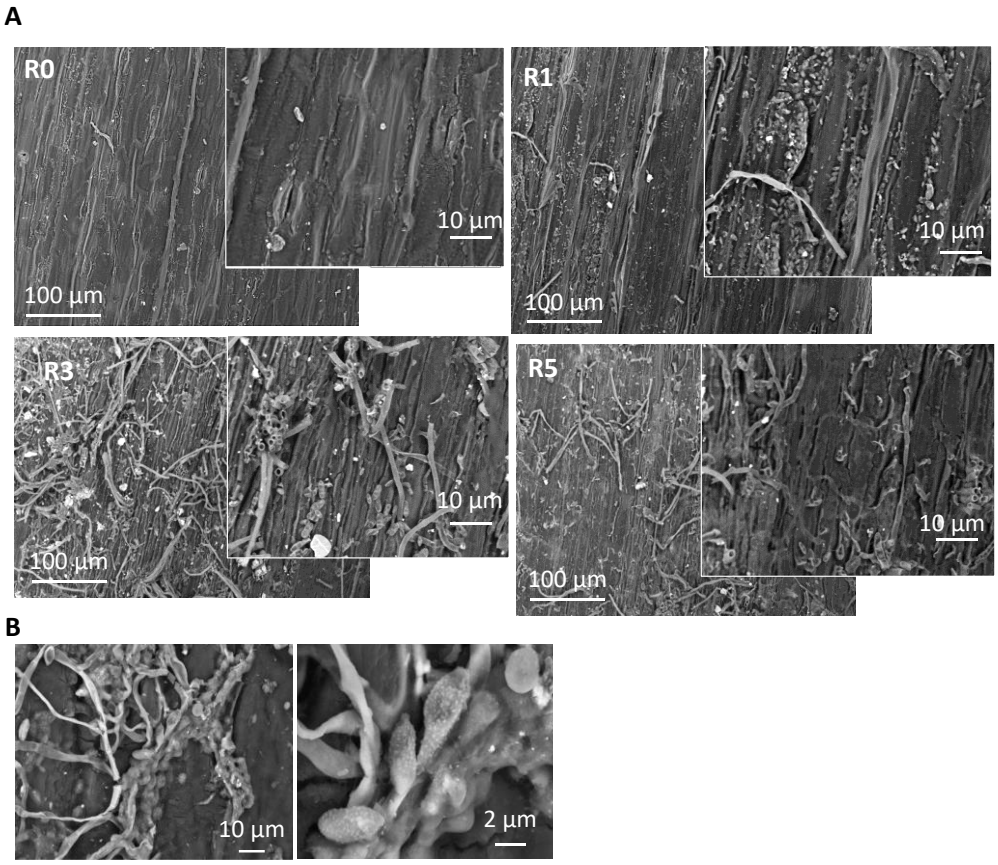

Figure S2: Scanning electron micrographs of the stem surface showing gradual microbial colonization at the different stages of dew-retting (A) and the association of bacteria and fungi to form a mixed bacterial- fungal biofilm (B)

Figure S3

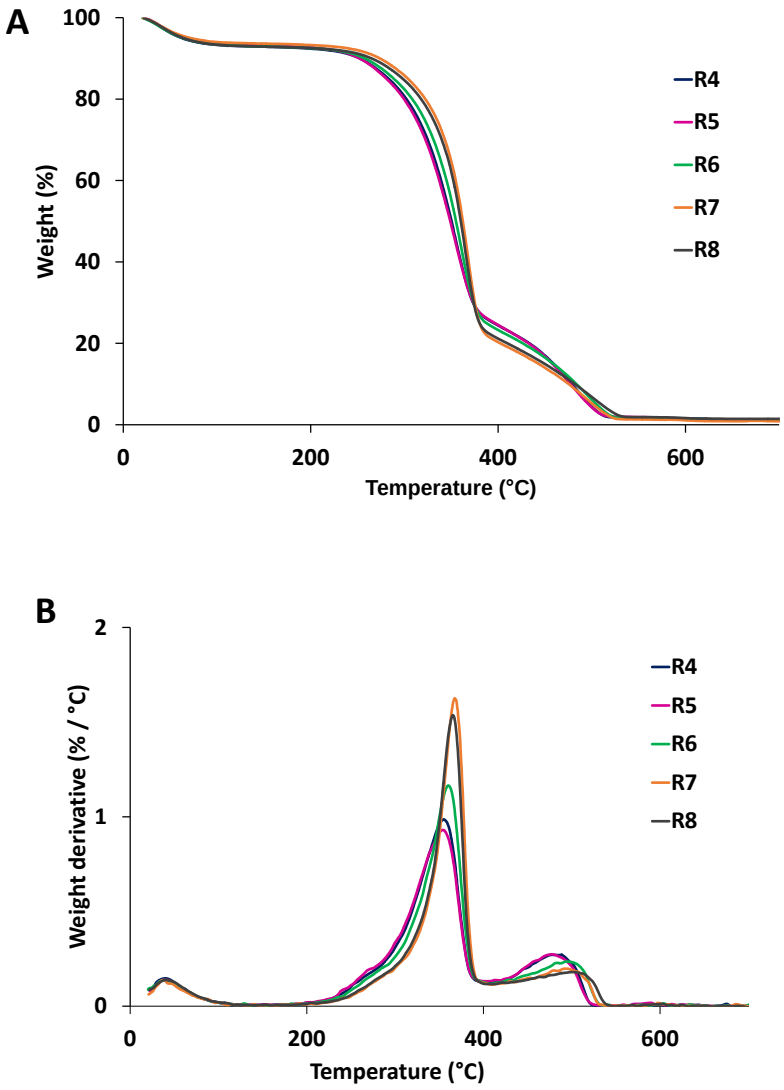

Figure S3: TGA thermographs and derivative thermographs of flax technical fibers extracted at different retting stages

Table S1: Bacterial alpha-diversity in all samples during dew-retting

Table S2: Fungal alpha-diversity in all samples during dew-retting

Table S3: Bacteria OTU table listing all OTUs detected and their abundance normalized by a subsampling

Table S4: Fungi OTU table listing all OTUs detected and their abundance normalized by a subsampling