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Influence of field retting on physicochemical and biological properties of “Futura 75” hemp stems

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A B S T R A C T

The current development of “*Cannabis sativa* L.” hemp fibers for technical textiles and biocomposites demands through consideration of the retting process's effect on fiber quality. Field retting, though crucial, remains insufficiently managed due to its reliance on empirical methods and dependence to weather conditions. This study aims to improve the understanding of the retting process in the case of the “Futura 75” hemp stems harvested at the end of the flowering period and retted in southern France, which is characterized by a dry Mediterranean climate. Various analyses were performed, including color evolution, morphological (optical microscopy), surface (SEM), biological (density of bacterial and fungal community using qPCR approach), and biochemical (solvent extraction and spectro-colorimetry) analyses. The results showed that retting had a major impact on the chemical composition of the fibers, resulting in a significant loss of pectic substances. This result correlated with microscopic observations, which showed the decohesion of fiber bundles during retting. Since an increase in cellulose proportion was observed, this was associated with both the degradation of the non-cellulosic components and an increase in cellulose crystallinity. A color change from green to black during retting, mainly due to microbial colonization was also observed. This result was further supported by the qPCR results, which indicated an increasing presence of bacterial and fungal populations on the hemp stems during the retting process. All the obtained results are statistically analyzed to identify correlations that, in the long term, could eventually lead to the identification of simple indicators to better control the field retting of hemp and obtain stable fiber properties. The statistical findings suggest correlations between variables related to microbial community, pectin degradation, and climatic data throughout the retting process.

Keywords:

Hemp fibers

Retting

Bacterial and fungal communities

Biochemical composition

Stem microstructure

Correlation analysis

1. Introduction

In the context of sustainable development and eco-conception, which aims to limit the impact of synthetic fiber-based materials on the environment and human health (Zheng et al., 2022), the use of natural fibers (hemp, flax, jute, ...) as substitutes for synthetic fibers (glass, carbon, and metallic fibers) is becoming more widespread (Charlet, 2010).

Lignocellulosic fibers derived from flax “*Linum Usitatissimum* L.” and industrial hemp “*Cannabis sativa* L.” have specific mechanical properties competitive with those of synthetic fibers. They are available, low-cost, and biodegradable (Beaugrand et al., 2014; Bourmaud et al., 2020). Therefore, they are used in several applications such as paper industry, thermal insulation, textile, fabrics, and furniture applications, and as

composites and plastic alternatives, etc. (Kymäläinen and Sjöberg, 2008; Muzyczek, 2020; Kulma et al., 2014; Crini et al., 2020). These fibers can be effectively used to produce bio-composites for various industrial applications, such as building and transportation industries (Murali et al., 2022), especially to reduce carbon dioxide emissions. The hemp and flax fibers can also be considered as an alternative to cotton fibers due to their generally lower ecological footprint, considering factors like pesticide use, irrigation requirements and land use (Duque Schumacher et al., 2020; De Vos et al., 2022).

Recently, the flax fiber market has become saturated as the development of plant-based materials has advanced significantly, resulting in an increasing demand for flax fibers. Flax crops require special climatic (low temperatures and high humidity) and cultural (with flax cultivated

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on the same land only once every six to seven years) conditions (Grégoire et al., 2021). Thus, traditional flax production areas have therefore reached the limits of their production capacity (Deng and Tian, 2015). At the same time, the demand for natural resources is increasing due to increasing industrial activities. Moreover, the current transition to a biobased economy requires additional biomass production, especially lignocellulosic biomass (Loeffler et al., 2017). It is, therefore, necessary to find other biomass crops to increase alternative sources of natural fibers.

The hemp plant has recently been attracting increasing interest worldwide. Hemp, native to Asia, is a dicotyledonous plant belonging to the *Cannabaceae* family and the genus *Cannabis* (Crini et al., 2020). It is considered one of the oldest domesticated crops in the world, cultivated in many countries in Asia and Europe. In addition, North America also has a rich history of hemp cultivation, dating back to the mid-18th century (Rupasinghe et al., 2020). However, legal restrictions significantly slowed production in the 20th century. Recent years have seen a revival of interest and renewed legal cultivation in North America (Adesina et al., 2020). In recent years, the area dedicated to hemp cultivation has increased significantly, reaching 75% from 2015 to 2019 (European Commission website, 2019). Hemp is an eco-friendly and fast-growing crop, which is characterized by drought resistance, less fertilizer than many commercial crops like wheat, maize, or cotton, the absence of the use of pesticides, and the ability to produce high biomass yields (Piotrowski and Carus, 2011; Duque Schumacher et al., 2020; De Vos et al., 2022; Tang et al., 2022). In addition, phytoremediation, particularly using industrial hemp, presents a promising approach due to its ability to sequester pollutants via its root system, making it an asset in circular economy models aimed at sustainable soil management (Golia et al., 2023). The entire hemp plant (stems, inflorescences, and seeds) is used to produce numerous products in various industrial fields, specially building materials, automotive, nutritious food and beverages, and cosmetics (Crini et al., 2020; Tang et al., 2022).

The general structure of the hemp stem has already been described by several authors (Schäfer et Honermeier, 2006; Morin-Crini et al., 2019). The hemp stem, in a transversal section, from the outer to the inner part, is composed of (i) an outer layer of the thin epidermis, a hypodermis, and a chlorenchyma, (ii) a layer of fiber bundles (primary bast fibers layer followed by a secondary bast fibers layer), (iii) the cambium, and (iv) the woody core (Amaducci et al., 2015). The fibers located in the phloem are assembled in bundles containing elementary fibers. Parenchyma cells separate the bundles of primary bast fibers (long fibers) and secondary bast fibers (short fibers). Elementary fibers are connected by their middle lamella, which is considered an amorphous layer composed mainly of pectin (Fuentes et al., 2017; Morin-Crini et al., 2019).

Hemp fibers offer economic valorization potential due to their various applications (Beaugrand et al., 2014; Sen and Reddy, 2011). Therefore, new opportunities for hemp raw materials (technical fibers, oils) have emerged, in addition to its traditional uses in the manufacture of rope, and paper (Placet et al., 2017).

Hemp grows under a wide range of pedoclimatic conditions in most areas of France and Europe (Grégoire et al., 2021). France is the largest producer in the European Union, accounting for over 70% of production with a cultivation area of around 12,000 ha in 2019 (Morin-Crini et al., 2019; European Commission website, 2019). In France, hemp is cultivated mainly in northern France (especially in Normandy, the origin flax cultivation area). However, it is a plant that can also be grown in other regions such as southern France, even if these regions are increasingly affected by global warming because hemp can resist drought. Hemp fibers can thus satisfy the growing industrial demand for natural products and would open a complementary market for flax fibers (Grégoire et al., 2021). However, the potential of hemp fibers to meet industrial demand for fiber-based materials is limited by the lack of accurate information on how to control the intrinsic properties of the fibers (Müssig and Amaducci, 2018).

From seed to fiber, hemp plants undergo several transformations. This begins with planting (sowing) in the field. Following a period of growth, the mature plants are harvested, marking the beginning of the next stage. Then comes retting, a natural process that allowing easier mechanical extraction of the fibers. Finally, through post-harvest processing steps such as scutching, hackling, spinning, weaving and potentially further processes depending on the final use, the extracted fibers are gradually transformed into a diverse range of products. All these steps affect the final properties of the fibers, especially the retting process. Thus, plant fibers exhibit differences in some of their characteristics (chemical and physical) that affect their mechanical properties and therefore limit their use in industrial applications where high fiber stability and homogeneity are required (Musio et al., 2018). These variations also influence fiber morphology and determine the adhesion mechanisms between plant cell walls and thermoplastic or thermoset matrices, particularly in the production of polymer composites (Bourmaud et al., 2018). In addition, it should be noted that the properties of the fibers, present natural variations within each plant, regardless of the variety, the cultivation techniques, or the fiber transformation performed as shown, for example, in Amaducci et al. (Amaducci et al., 2015).

Field retting is a traditional biological process that contributes to the decohesion of fiber bundles, which is necessary for improving the mechanical properties of the fibers (Mazian et al., 2019). In Europe, field retting is performed in many different regions because it is inexpensive and easy to apply compared to other alternative methods (Réquillet et al., 2021). After harvesting, hemp stems are laid out on the soil and colonized by microorganisms naturally present in the soil and on the stems. During retting, the hydrolytic enzyme activities produced by stem-colonizing microbial communities (bacteria and fungi) provide, firstly, the degradation of the parenchymatous cells linking the fiber bundles and ensure progressively their separation from the surrounding stem tissues, and, secondly, a reduction in the cohesion between the fibers by degrading the middle lamellae linking the elementary fibers (Djemiel et al., 2017).

The effectiveness of the retting process depends strongly on climatic conditions (temperature and humidity), the farmer's experience, and the duration of the retting (Mazian et al., 2018). Then, problems of the inconsistency of fiber quality (fiber fineness, tensile strength) can occur and are considered an issue for industrial applications.

Therefore, in this study, the effects of the duration of field retting and climatic conditions on the properties of hemp fibers retted in a "non-ideal zone" (south of France) are investigated. This will help us to determine the temporal dynamics of the retting process in a warm Mediterranean climate. During the retting process, both qualitative and quantitative experimental techniques were used to study the composition and structural properties of Futura 75 variety stems and fibres. We employ a multidisciplinary approach, combining visual observations, bio- and physico-chemical analysis, and microscopic observations. Finally, we used an original correlation approach that investigates the relationship between climatic data and intrinsic characteristics of hemp fibers during the retting process. The goal is to enhance the understanding of retting and ultimately optimize its control, aiming to produce high-quality fibers suitable for diverse industrial applications.

2. Materials and methods

2.1. Raw material

2.1.1. Cultivation

Hemp plants (*Cannabis sativa*, Cultivar Futura 75) were sown at a rate of 40 kg/ha on April 15, 2021, in the south of France in the Drôme Chanvre Association (<http://www.dromechanvre.fr/>) (Mirabel et Blancs, France, 44° 42' 35"N, 5° 05'29"E). The characteristics of this plant, including a height of approximately 2 m and a diameter of 6 ± 0.5 mm, were determined through measurements taken from over thirty

individual stems. In addition, based on literature, this hemp variety is considered as a high-fiber variety (Vandepitte et al., 2020). The hemp plants were harvested at the end of flowering (September 2, 2021) using a sectional mower adapted to this crop. At harvest, the stems were cut off at the base and discarded on the ground.

2.1.2. Implementation of retting and field instrumentation

After harvest, hemp plants were transported to Mas de La Valus (Bouquet, France, 44°09'52"N, 4°16'56"E) for logistical reasons (close to the laboratory site) and retted in the field. Plants were cultivated and retted on similar clayey gravelly soils (<https://www.geoportail.gouv.fr/donnees/carte-des-sols>) with a slightly basic pH (8.1 at Mirabel et Blacons and 8.4 at Mas de la Valus).

Hemp plants were retted from September 3, 2021, until October 17, 2021. The swaths were defined according to a non-systematic W pattern shown in Fig. 1. Five swaths (250 stems/swath) were retted according to the weather conditions. Plants were turned regularly (once a week) to homogenize the stems' retting and obtain a more uniform fiber dissociation.

2.1.3. Sampling and weather conditions

Replicate stems and soil samples were collected from the 5 swaths weekly at regular intervals for 6 weeks (named R0 for unretted samples, R1 to R6 for retted samples during 1–6 weeks). For stem samples, the middle part (1 m long) was selected. The location of the fibers on the stems (top, middle, and bottom) affects the quality of the fibers. The fibers from the middle part of the stems generally have higher mechanical properties (strength, stiffness) than the fibers from the upper and lower regions (Charlet et al., 2010, Liu et al., 2015a). In addition, studies have shown that selection of the middle part of the stems could reduce the heterogeneity in the original chemical composition of the stems, which differs along the stems (Crônier et al., 2005; Liu et al., 2015a; Bleuze et al., 2018).

A part of the stem samples dedicated to biological analysis was stored directly at -80°C , while the other part of the stems was stored at room temperature. Soil samples were collected from the plot where the retting was implemented, at a depth of 20 cm, sieved (pore size < 1.0 mm), freeze-dried, and stored at -80°C .

A study (Réquillé et al., 2021) has shown that the adequate level of retting depends mainly on the level of rainfalls. Thus, climatic data during the retting period was obtained from Météo France (<https://publitheque.meteo.fr>) from a meteorological station (Méjannes-le-Clap, France, 44°13'19"N, 4°20'41"E) at 13 km from Mas de la Valus, where the retting was carried out. The main climatic variables collected are the average air temperature, the relative humidity, and the daily rainfall. In addition, temperature, and humidity sensors (Hygro Bouton 23, Plug&Track, Lille, France) were placed on the soil surface to regularly monitor these two parameters during retting.

2.2. Experimental methods

2.2.1. Visual aspects

The color of the stem surfaces and fibers (extracted manually) was

visually evaluated during the retting process.

2.2.2. Microscopic observations

The unretted (R0) and retted stems (R2, R4, and R6) were examined with a Scanning Electron Microscope (SEM, FEI Quanta 200 FEG, Netherlands). The effect of field retting duration on fiber morphology was also studied using SEM and optical microscopy (Leica Laborlux 11 POL S) equipped with a 1600×1200 pixels mono-CDD Sony digital camera.

Before optical microscopy observation, cross sections of the stems were cut with a circular saw, coated with an epoxy resin (Presi, Eybens, France), and then polished using different fine polishing papers (600, 2400, and 4000 grades). Images were taken using Archimed® software to analyze qualitatively and quantitatively the evolution of the different components of the hemp stem (epidermis, fiber bundles, and xylem) during field retting.

2.2.3. Biochemical analysis

2.2.3.1. Analysis of cellulose, hemicellulose, waxes, lignin, and ash. The biochemical composition of hemp fibers was determined by successive solvent extractions. Tests were performed according to different American Society for Testing and Materials (ASTM) standards (Fig. 2). The mass percentages of the various components of the fiber cell wall (cellulose, hemicellulose, lipophilic extracts, ash) were calculated. Fibers were manually extracted from the stems to within 10 g (dry weight) and cut into fine pieces (1–2 mm in size). The fibers were dried at 105°C for approximately 12 hours before performing the biochemical analysis. The details of the chemical products used in the biochemical analysis are provided in a supplementary table (Supplementary table 1).

• Lipophilic extract proportion

ASTM method D1107–96 covers determining the ethanol-toluene soluble proportion of the fibers, which measures the waxes, fats, resins, oils, and some gums, as well as some water-soluble substances. The dried fibers were treated for 8 h with 150 ml of an ethanol-toluene mixture (1:2 w/w) using a Soxhlet extraction apparatus. The residue was then filtered, dried at 105°C , and cooled before being weighed using an infrared balance (Precisa XM66, Precisa, Dietikon, Switzerland).

• Holocellulose proportion

Holocellulose is composed of both hemicellulose and alpha-cellulose. The holocellulose proportion of fibers was determined according to standard D 1104–56. A quantity of 2.5 g of the fibers obtained after treatment with the ethanol-toluene mixture were introduced in a solution containing 80 ml of distilled water, 0.5 ml of glacial acetic acid ($\text{C}_2\text{H}_4\text{O}_2$), and 1 g of sodium chlorite (NaClO_2). The mixture was heated at 75°C for 5 h with stirring. Every hour, 0.5 ml of glacial acetic acid and 1 g of NaClO_2 were added. After 5 h, the mixture was placed in an ice water bath until the temperature of the solution reaches 10°C . The residue was then filtered and washed with distilled water (the color changed from yellow to white). The

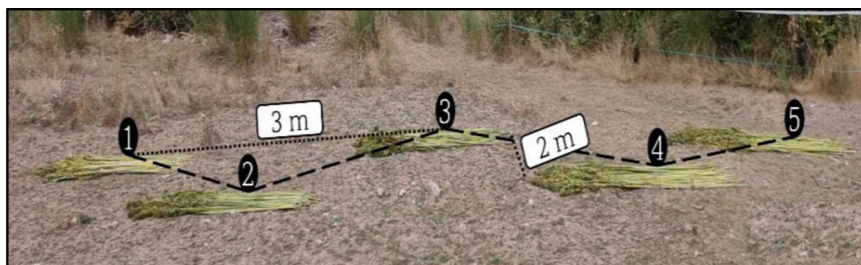


Fig. 1. Experimental site (Mas de la Valus) during the field retting campaign.

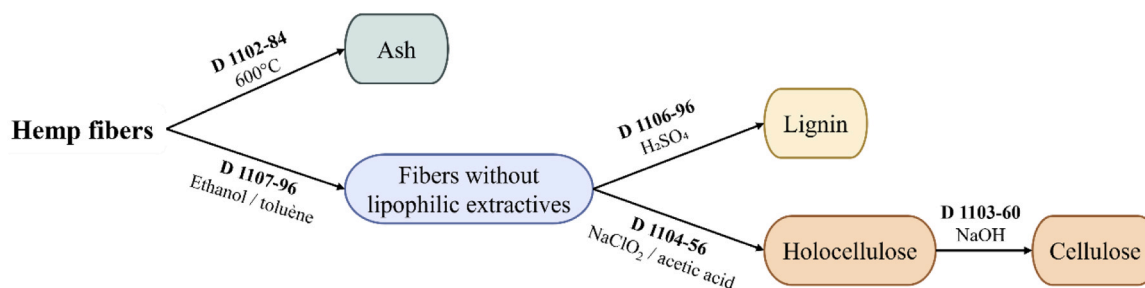


Fig. 2. Schema of successive solvent extractions (according to the ASTM standards) used to analyze the biochemical composition (lipophilic extracts, lignin, holocellulose, cellulose, and ash) of hemp fibers.

residue was dried in an oven at 105°C and then weighed with an infrared balance at 105°C.

• **Cellulose proportion**

According to ASTM method D1103–60, 2 g of dried holocellulose from the previous test was treated with 25 ml of a 17.5% (w/w) sodium hydroxide (NaOH) solution for 30 min. Then 33 ml of distilled water was added, and the mixture was stirred for 1 h. The residue was filtered and washed with an 8.3% (w/w) NaOH solution, 10% acetic acid, and distilled water. The residue was dried in an oven at 105°C and then weighed with an infrared balance at 105° as alpha-cellulose.

The hemicellulose proportion was determined by subtracting the alpha-cellulose proportion from the holocellulose proportion.

• **Lignin proportion**

A quantity of 1 g of the fibers obtained after treatment with the ethanol-toluene mixture were stirred in a 72% H₂SO₄ solution for 2 h with frequent stirring. This method aims to hydrolyze the carbohydrates and obtain an insoluble residue, which was determined as Klason lignin according to ASTM method D 1106–96. The mixture was then diluted by adding distilled water to obtain an H₂SO₄ solution with a concentration of 3% before boiling the mixture for 4 h under a reflux condenser. The residue was then filtered and washed with distilled water, dried at 100°C, and weighed.

• **Ash proportion**

A quantity of 2 g of dried fiber was heated to 600°C for 8 h in an oven (dry oxidation), then cooled at room temperature. The residue was weighed to determine the change in fiber weight according to ASTM D 1102–84.

2.2.3.2. *Pectin analysis.* The determination of evolution of the pectin proportion during retting was determined using a colorimetric method (Blumenkrantz et al., 1973; Van Den Hoogen et al., 1998). The amount of pectins for unretted fiber samples (R0) and retted samples after 1 (R1), 2 (R2), 3 (R3), 4 (R4), and 6 (R6) weeks were evaluated. For each week of retting, all 5 swaths were examined.

This method consists of the hydrolysis of the pectic substances to galacturonic acid, the main compound of pectin, followed by a coloring step with m-hydroxydiphenyl. A color complex between the reagent and

the galacturonic acid is produced (Fig. 3).

The standard assay was performed using different amounts of commercial pectin (Coprare, Alès, France) ranging from 0 to 8 µg (0, 0.5, 1, 1.5, 2, 4, 6, and 8) in 40 µl of distilled water (i.e., a concentration of 0–200 µg ml⁻¹) in 96-well microtiter plates. After adding 200 µl of concentrated sulfuric acid H₂SO₄ (96%) to each well containing 40 µl of pectin, the sample and reagent were mixed, and the plate was incubated at 80°C for 1 h. After cooling to room temperature, the absorbance of the samples was measured at 540 nm using a spectrophotometer (Thermo Scientific Specter Multiskan®, Waltham, MA USA). Then, 40 µl of m-hydroxydiphenyl (100 µl of 100 mg of m-hydroxydiphenyl in 1 ml of dimethylsulfoxide mixed with 4.9 ml of 80% sulfuric acid) was added to all wells. After 15 min, a pink color appears, and the absorbance was measured again at 540 nm. The proportion of galacturonic acid was determined by subtracting the two absorbance values. A calibration curve between 0 and 8 µg of galacturonic acid was generated and will be used to determine the pectin proportion in the fiber samples.

To determine the amount of pectin in the fiber samples, each fiber sample of 200 mg (dry weight) was cut into pieces 0.5–1 mm long. The lipid components were removed by extraction with 2 ml chloroform/methanol (1:1 v/v) solution under stirring at room temperature for 24 h. After drying at 105°C, the pectins were extracted with 200 ml of 5 g.l⁻¹ Na₂EDTA for 2 h at 100°C. Then, 40 µl of this solution was used to perform the first absorbance reading after the addition of H₂SO₄ (as described above). The second reading absorbance of the samples was measured at 540 nm after adding m-hydroxydiphenyl solution. Finally, pectin proportion was determined by subtracting the two absorbance values from the initial calibration curve.

2.2.3.3. *Cellulose crystallinity.* Cellulose crystallinity was determined by X-ray diffraction measurements performed on cut and compressed fibers. Dry fibers (1 g) were cut into small pieces (< 1 mm) and cold pressed (30 bar) to obtain disks of 25 mm in diameter and 2 mm in thickness. The unretted fibers (R0) and retted fibers (R2, R4, and R6) were analyzed in triplicates using an X-ray diffractometer (XRD, AXS D8 Advance Bruker) equipped with Cu-Kα radiation (λ = 1.54 Å). X-ray diffractograms were collected from 2θ = 5–70° with a scanning speed of 0.01°/s. Using a deconvolution method, the crystalline order index (CI) was determined from X-ray diffractograms. Individual crystalline peaks were fitted using Origin® software assuming Gaussian functions. CI was calculated from the ratio of the area of all crystalline peaks (101), (101)

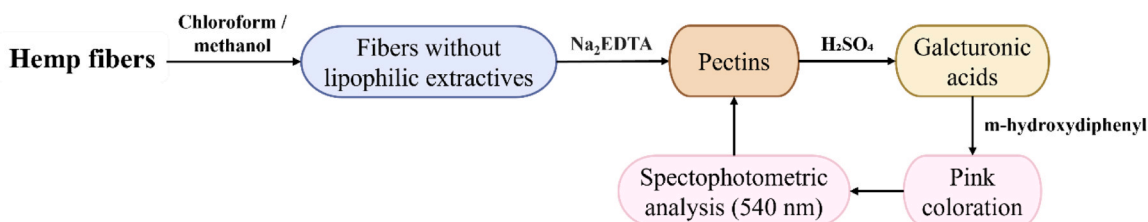


Fig. 3. Schema showing the different steps to determine the pectin proportion in hemp fibers.

and (002) to the total area according to the following equation: $CI = [I_c / (I_c + I_{am})] \times 100\%$, where I_c : crystalline peak areas ((101), (10 $\bar{1}$) and (002)) and I_{am} : amorphous broad peak area.

2.2.4. Evolution of bacterial and fungal communities during retting

2.2.4.1. Extraction and purification of genomic DNA from soil and stem samples. During retting, DNA was extracted from Futura 75 stems (R0, R1, R2, R3, R4, and R6) and soil samples (R0, R2, and R4). The DNA was extracted from stem and soil samples of the 5 different swaths.

DNA extraction was performed following the protocol of the Genosol platform (INRAE, Dijon, France) (Internal reference: G.MO-026.6, 2020) (Lelievre, 2020; Bou Orm et al., 2023). Purification of the extracted DNA was performed using the Nucleospin Soil Kit (Macherey Nagel, Düren, Germany) and was performed according to the manufacturer's instructions.

The amount of extracted DNA was determined by measuring absorbance at 260 nm using Nanodrop technology. Subsequently, the extracted DNA was also quantified by measuring absorbance at 535 nm after PicoGreen staining (Quant-iT™ PicoGreen™ dsDNA Assay kit, Thermo Fisher, Bleiswijk, Netherlands). This accurate quantification was used to further determine the abundance of bacterial and fungal communities by qPCR (quantitative polymerase chain reaction).

DNA purity level was determined using the NanoDrop™ spectrophotometer (ThermoScientific, Wilmington, USA) by measuring the absorbance of 1.5 μ L of extracted DNA at 280 and 230 nm and by determining the ratios of the 260/280 nm and 260/230 nm values.

2.2.4.2. Fungal and bacterial population density levels. Quantitative real-time PCR was used to determine the abundance of bacteria and fungi in each sample by examining the 16S rDNA (bacteria) and 18S rDNA (filamentous fungi) genes. All DNA extracts were amplified in triplicate. The efficiency of the qPCR performed was above 90% for all samples (Bou Orm et al., 2023).

2.2.4.3. Fungal 18s rDNA. The primers used for targeting the fungal community are nu-SSU-0817-F (5'-TTAGCATGGAATAATRAATAGG A-3', with R = A or G, Tm 54.2°C) and nu-SSU-1196-R (5'-TCTGGACCTGGTGAGTTTC-3', Tm 56.7°C) (Borneman and Hartin., 2000). The Hot Firepol EvaGreen kit (Solis BioDyne, Tartu, Estonia) was used in the Corbett Research Rotorgene 6000 thermal cycler (QIAGEN, Hilden, Germany) to perform quantifications according to the following program: initial activation at 95°C for 15 min followed by 48 cycles of denaturation at 95°C for 18 s, hybridization at 60°C for the 60 s and extension at 72°C for 20 s. A PCR product of 422 bp is expected (Bou Orm et al., 2023).

2.2.4.4. Bacterial 16s rDNA. Primers used to target the bacterial community are BAC338F (5'-ACTCCTACGGGAGGCAG-3', Tm 57.6 °C), BAC805R (5'-GACTACCAGGGTATCTAATCC-3', Tm 57.9 °C) and the probe BAC516F (FAM-TGCCAGCAGCCGCGGTAATAC-TAM) (Yu et al., 2005). The qPCR was performed using the GoTaq Probe qPCR Master mix kit (Promega, Madison, USA) in a Corbett Research Rotorgene 6000 thermal cycler (QIAGEN, Hilden, Germany) according to the following program: initial activation at 95 °C for 4 min followed by 50 cycles of denaturation at 95 °C for 15 s and hybridization-extension at 60 °C for the 60 s. A PCR product of 468 bp is expected (Bou Orm et al., 2023).

2.3. Statistical analysis

Spearman rank correlation coefficients were calculated to look for any significant correlations between climatic conditions (temperature and rainfall), the physicochemical properties of hemp fibers, and bacterial and fungal community density. Spearman correlation coefficients are computed as the standard Pearson correlation coefficients but

applied to the point's ranks rather than to their values. In the same way as Pearson correlation represent the linear links between variables, Spearman correlations represent the monotone links between variables. This extension can be of high value for many life variables that are correlated but not necessarily linearly (de Winter et al., 2016).

3. Results and discussions

3.1. Evolution of weather conditions during retting

Rainfall is not evenly distributed during the 6 weeks of retting: less than 10 mm during the first two weeks of retting, heavy rainfall (> 93 mm) after 3 and 4 weeks, and no rainfall during the last 2 weeks of retting (Fig. 4.A).

When comparing the rainfall levels in September-October 2021 with the data from the past 10 years, it is evident that the distribution of precipitation has consistently been heterogeneous. However, in 2021, the rainfall level reached 368 mm, exceeding the average value of 107 ± 73 mm observed over the past decade (<https://publitheque.meteo.fr>). Thus, the retting period experienced significantly higher rainfall compared to previous years.

In contrast to the rainfall distribution, humidity is evenly distributed during the retting period and averaged about 71% (Fig. 4.A). By examining the average relative humidity values for the same months as our retting campaign (September-October) over the past 10 years (2011–2020), it is noticeable that the humidity levels have consistently remained high, with an average of $69 \pm 8\%$.

Average maximum and minimum temperatures showed a parallel decreasing trend between weeks 1 and 6 (Fig. 4.B). Maximum and minimum temperatures ranged from 31 to 16°C and 19–4°C, respectively. These values could lead to the development of a microbial biofilm favored by uniform temperatures (around 20°C), as it is known that the metabolic activity of microorganisms may depend on the temperature values (Robador et al., 2016).

Since it is a biological process, retting requires both important moisture and temperatures warm enough for microbial colonization, development, and activity (Ehrensing, 1998; Djemiel et al., 2020). Therefore, the observed conditions (temperature, humidity, and rainfall) are considered favorable for the retting process.

Average soil temperature showed a parallel decreasing trend between weeks 1 and 6 (Fig. 4.C). During retting, the temperature varied between 24 and 12°C. Soil humidity is not evenly distributed during the retting period: in the first week, a decrease is observed from about 90–50%, then an increase is observed (70–100%) after R1. This value can be explained by the clay loam soil which had a high-water holding capacity (Réquillé et al., 2021). It should also be noted that the hygro-buttons sensors may be saturated as a result of heavy precipitations after R2.

The variability in weather conditions during the retting process, particularly in terms of rainfall, may affect the quality and efficiency of the retting process. However, the lack of rainfall during the last two weeks of retting could potentially improve the final fiber properties by preventing over-retting and loss of fiber strength (Henriksson et al., 1997; Dey et al., 2021). Furthermore, optimal humidity levels of 70% and temperatures ranging from 12 to 25°C may be suitable for the retting process, as these conditions have been observed to promote the growth and activity of microorganisms on the surface of the stems (Djemiel et al., 2020).

3.2. Influence of field retting duration on the color of hemp stems and fibers

Fig. 5 shows the photographs of the stems and the processed fibers. After a visual analysis of the hemp stem and fibers, an important change in color is marked. The color of the stem, which is light green before retting (R0), becomes pale gray and shows increased black spots after

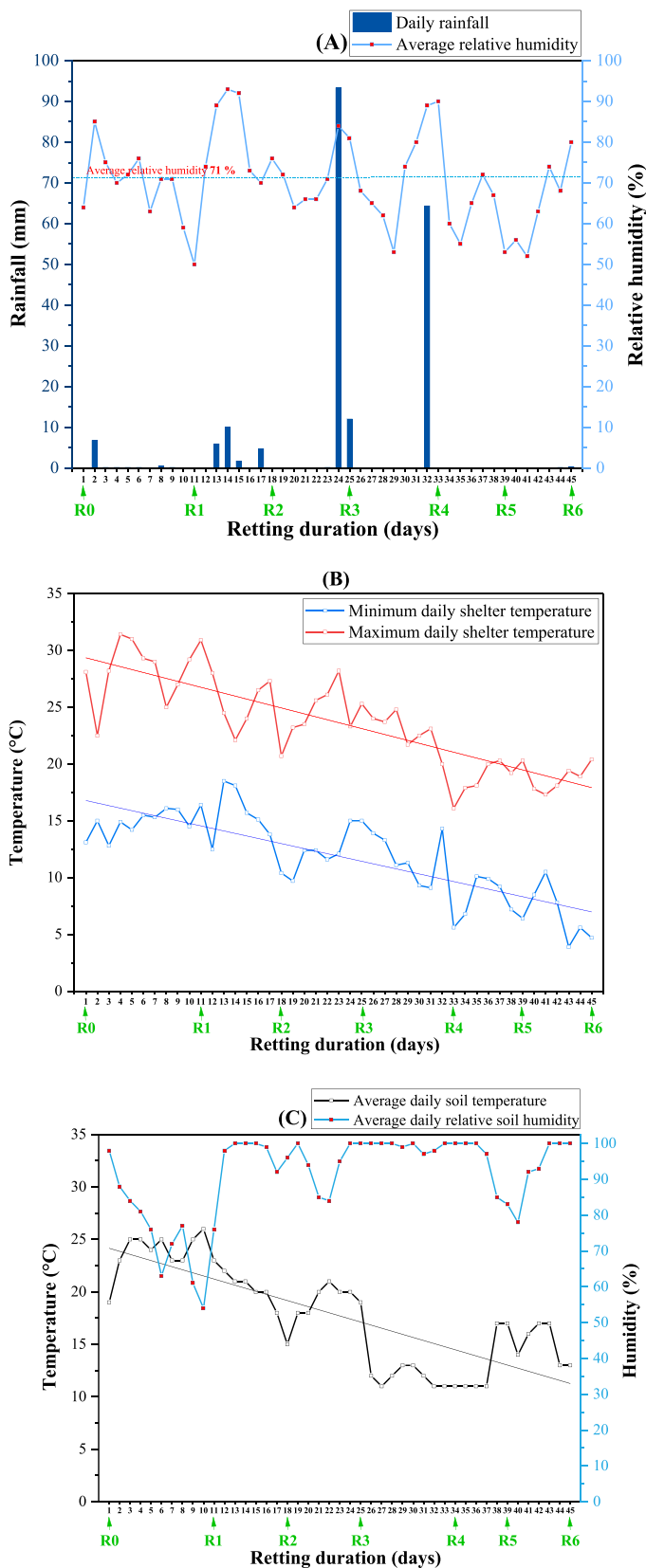


Fig. 4. Relative air humidity and rainfall (A), minimum and maximum air temperature (B), average daily soil temperature, and soil humidity (C) during the field retting process of hemp stems.

two weeks of retting (R2). After four weeks (R4), stems become dark gray with extensive black spots. Finally, after six weeks (R6), the stems become black. These color changes can be explained by the degradation of plant pigments responsible for the green color (chlorophyll). It depends on climatic conditions and occurs through abiotic and biotic mechanisms such as photodegradation, chemical modifications, microbial colonization, and biodegradation (Mazian et al., 2018; Bleuze et al., 2020). Significant changes in the stem appearance have been described for hemp field retting (Jankauskiene and Gruzdeviene, 2013; Placet et al., 2017; Mazian et al., 2018) and the link between the occurrence of the black color of the stems and the metabolic activities of microorganisms especially fungi has been reported (Akin et al., 2000; Ribeiro et al., 2015). This qualitative analysis can give an idea of the evolution of retting by classifying the stems according to the progress of their retting degree.

The fibers have the same color as the stems from which they were extracted. These color changes in the fibers can be explained by the colonization of the epidermis by active functional fungal and/or bacterial populations.

3.3. Morphological changes of hemp stem during retting

3.3.1. Microscopic imaging of the stem tissues

A cross-section illustrating the structure of an unretted hemp stem (R0) obtained with an optical microscope is shown in Fig. 6.A. For each component of the stem, 60 measurements of thickness were taken for unretted (R0) and retted hemp stem (R4) (Fig. 6.B and C).

The main components of the hemp stem are the central cavity ($1500 \pm 49 \mu\text{m}$), the xylem ($1008 \pm 161 \mu\text{m}$), and the surrounding cortex, which contains fiber bundles (including both primary fibres, $74 \pm 16 \mu\text{m}$, and secondary fibres, $19 \pm 6 \mu\text{m}$). The outside of the stem is covered with the epidermis ($10 \pm 3 \mu\text{m}$).

A significant increase ($p\text{-value} \leq 0.05$) in fiber bundle thickness is observed after 4 weeks of retting (R4) (primary fiber, $84 \pm 18 \mu\text{m}$ and secondary fiber, $33 \pm 7 \mu\text{m}$). However, no significant variation in the thickness of the stem components (xylem and central cavity) is observed during the retting process ($p\text{-value} \geq 0.05$).

3.3.2. Microscopic observations of hemp bast fibers during retting

The impact of retting on the morphology of hemp fiber is qualitatively studied by direct microscopic observations (Fig. 7). During retting, large variations in the morphology of hemp stems are observed. The morphological changes of the stems mainly affect the outer tissues, as previously shown (Chabbert et al., 2020). The individual fibers of the unretted stems (R0) appear to be well organized in bundles. Retted stems (R2 and R4) exhibit more decohesion within the fiber bundles compared with non-retted stems (R0). The parenchyma cells located under the epidermis and between the fiber bundles are gradually degraded, resulting in a few open spaces. This result confirms the positive effect of retting on decohesion within fiber bundles (Akin, 2013; Chabbert et al., 2020) and may explain the increase in fiber bundle thickness (primary and secondary) after 4 weeks of retting, as determined by microscopic observations.

To supplement the results obtained by optical microscopy, SEM images are obtained for unretted (R0) and retted hemp fibers (R2) (Fig. 8). The images show the surface of unretted fibers covered with amorphous materials that prevent observation of the cell walls. However, the retted fibers (R2) exhibited a cleaner surface, indicating that the amorphous materials could be removed during retting.

These changes in the morphology of hemp fibers during retting are mainly due to the action of microbial enzymes, which mainly target the wall sublayers rich in cellulosic fibers (Placet et al., 2017). The microbial activity would allow the removal of cell wall polysaccharides (pectin, hemicelluloses) and other cementing compounds that bind the fibers, resulting in the decohesion of the fiber bundles (Lashermes et al., 2016).

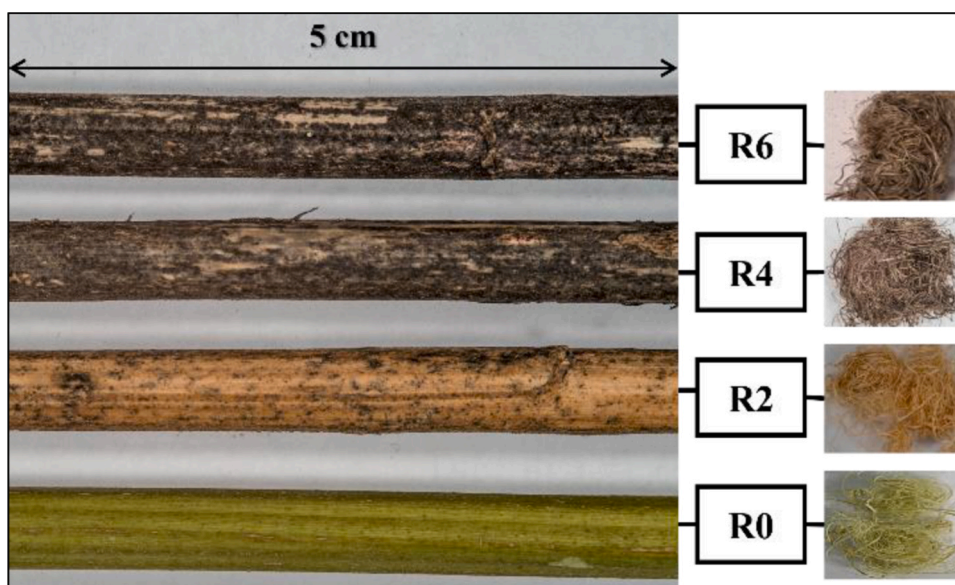


Fig. 5. Photos showing the evolution of the color of the stems and fibers during the retting process; unretted hemp stem (R0) and retted hemp stem for 2 (R2), 4 (R4), and 6 (R6) weeks.

3.4. Influence of field retting duration on the physicochemical properties of hemp fibers

3.4.1. Evolution of cellulose, hemicellulose, waxes, lignin, and ash proportions

It has been reported that plant age and variety, plant parts, fiber source, retting method, growing year, and climatic and geographical variations significantly affect the chemical composition of natural fibers (Lee et al., 2020; Jankauskiene et al., 2015). In addition, microbial colonization of the stem surface, during retting, leads to changes in stem composition, especially in the bast fibers (outer tissues). When microorganisms grow on a substrate, they first consume the absorbable compounds present in the soluble fraction and then produce enzymes that degrade structural cell wall compounds (pectin, hemicellulose, and lignin) to obtain the absorbable forms of nutrients (Lashermes et al., 2016).

Cellulose, hemicellulose, and lignin proportions play a significant role in determining fibers physical properties and potential applications. Retting is a crucial process that influences these component proportions (Müssig and Martens, 2003; Jankauskiene et al., 2015).

The evolution of the relative biochemical composition of hemp fibers as a function of retting time is shown in Fig. 9. The biochemical analysis was realized on unretted (R0) and retted hemp fiber samples after 2 (R2), 4 (R4), and 6 (R6) weeks. Duplicates were only performed for R0, R2, and R4 samples.

Cellulose is the main constituent of retted hemp fiber ($\approx 80\%$). A gradual increase in cellulose proportion (by $\approx 11\%$) is observed during retting from $68.4\% \pm 0.1$ (R0) to 79.2% at R6. These results show that after 6 weeks of retting, there was no decrease in cellulose proportion, indicating that there were no over-retting where microbial cellulolytic enzymes can lead to degradation of the cellulosic fibers (Réquillé et al., 2021). The cellulose proportion was stabilized after 4 weeks of retting ($77.7 \pm 1.0\%$). These results suggest that 4 weeks of retting might be the optimal duration to end the retting and that retting could be extended by a few weeks (2 in our case) without deteriorating fiber quality. Cellulose proportion has a significant impact on the mechanical properties of hemp fibers, especially on their tensile properties (Liu et al., 2015a). In our study on the Futura 75 hemp variety, an increase in cellulose proportion of 11% is observed during the retting process. Another study (Mazian et al., 2018), using the same experimental method, has shown an 18% increase in cellulose proportion after 9 weeks of retting for a

Santhica 27 variety harvested at the beginning of the flowering period. Another study performed on the Futura 75 field retting, using another experimental procedure, exhibits similar cellulose proportion of about 80.4% after 3 weeks of retting (Jankauskiene et al., 2015). However, according to another study (Liu et al., 2015b), performed on the USO-31 hemp variety harvested at the beginning of flowering and using another experimental method, the cellulose proportion exhibited an initial increase of more than 10% (from $\approx 70\%$ to more than 80%) after 50 days (≈ 7 weeks) of field retting process but subsequently decreased in later stages of retting. Even though all the studies demonstrate an increase in cellulose proportion during retting, it can be observed that cellulose proportion might change according to different factors such as hemp variety, retting duration, and experimental procedure.

Since the quantification of cellulose proportion is relative, thus its variation is related to the change in the proportion of other components of the plant wall. Hemicelluloses, which are non-cellulosic components, binding cellulose, and lignin together, act as cementitious substances of fiber bundles (Zimmiewska, 2022). A progressive decrease of about 50% in hemicellulose proportion is observed with retting time (11.1 ± 1.1 – 5.4% between R0 and R6). This decrease in hemicellulose proportion may lead to a change in the surface biochemistry of the fibers and an increase in fiber decohesion (Réquillé et al., 2021). However, in terms of mechanical properties, hemicellulose could have a lower contribution to the stiffness and strength of fibers (Reddy and Yang, 2005; Jankauskiene et al., 2015).

A study performed on the Futura 75 field retting, using another experimental procedure, exhibits similar hemicellulose proportion of about 6.4% at the end of the retting process (after 3 weeks) (Jankauskiene et al., 2015). Another study (Mazian et al., 2018), using the same experimental method, has shown a slight decrease (from 14% to 12%) in hemicellulose proportion after 9 weeks of retting for the Santhica 27 variety harvested at the beginning of the flowering period.

Ash (minerals) is considered a fibers minor component. During retting, ash proportion decreases by about 65% from $4.2 \pm 0.1\%$ at R0 to 1.5% at R6. A study performed on raw Futura 75 fibers shows a similar ash proportion (3.9%) (Bonatti et al., 2004). Another study performed on the field retting of the Futura 75 variety exhibits similar evolutions in terms of the percentage of plant wall constituents of hemp fibers (hemicellulose: 6.4% and ash: 2.1%) (Jankauskiene et al., 2015). When the ash proportion exceeds 2%, it can negatively impact the suitability of the fibers as pulp feedstock and can also affect the mechanical strength

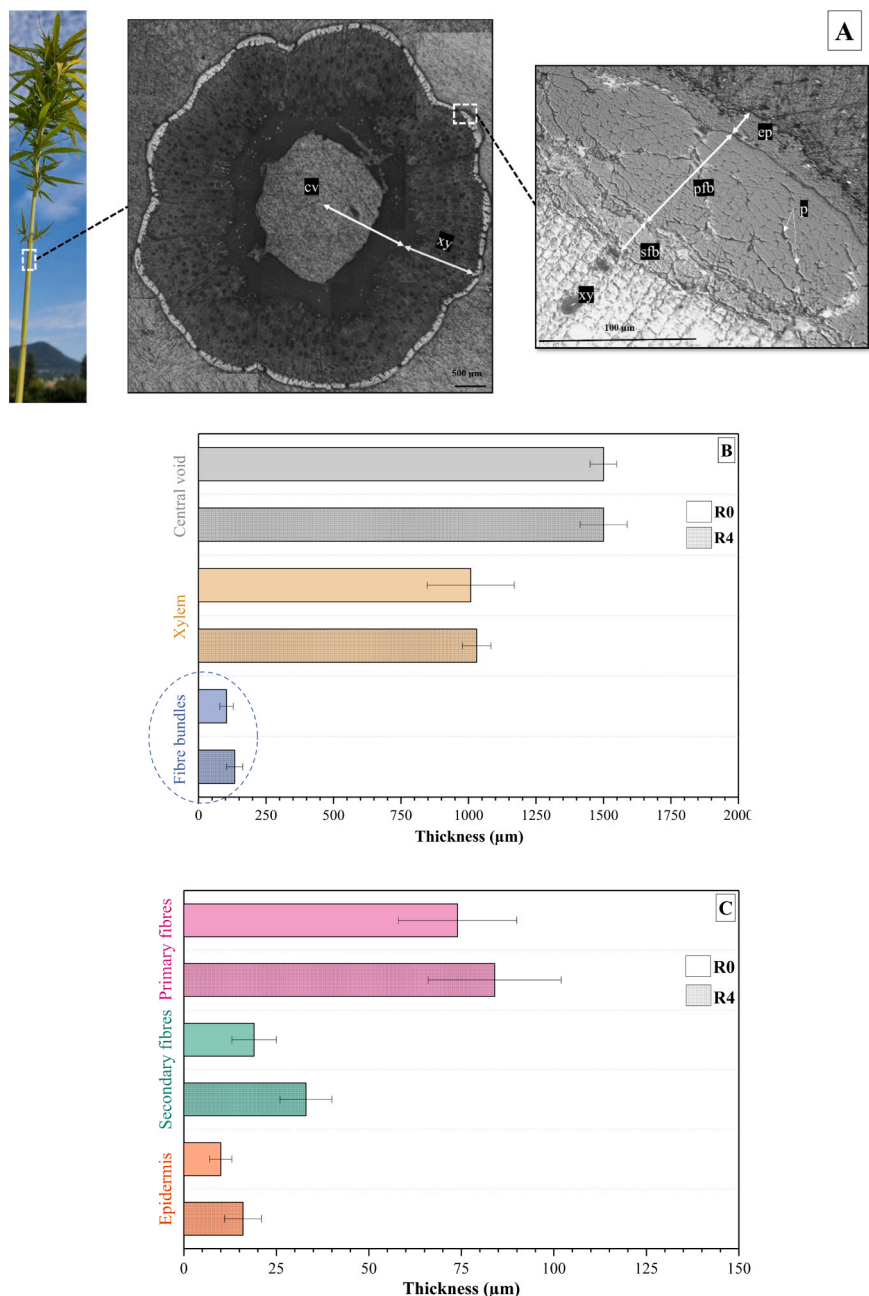


Fig. 6. Optical microscope micrographs showing a cross-section of an unretted hemp stem (A). Morphological characteristics (fiber bundle, xylem, and central cavity) (B) and (primary and secondary fibers and epidermis) (C) of hemp stem before retting (R0) and after 4 weeks of retting (R4); (cv) central void, (xy) xylem, (ep) epidermis, (pfb) primary fiber bundles, (sfb) secondary fiber bundles and (p) parenchyma. Scale bar = 100 μm .

properties of the final products (Jankauskiene et al., 2015).

Finally, lipophilic extractives proportion decreases from $6.0\% \pm 0.9$ at R0 to 1.7% at R6. The increase in cellulose proportion and decrease in lipophilic extracts and hemicelluloses during hemp field retting have already been reported in several studies (Nytker et al., 2008; Liu et al., 2015a; Mazian et al., 2018, 2019). By reducing the lipophilic extractives proportion, fibers become more suitable for various applications such as textiles, and paper (Singh and Singh, 2014). In addition, it could promote better interfacial bonding when incorporated within a polymer matrix to produce biocomposites. This may induce improved mechanical properties as observed by Mazian et al. in the case of polypropylene/hemp composites (Mazian et al., 2020).

Lignin represents a small proportion of the fiber cell walls. Lignin proportion increased from $2.0 \pm 0.3\%$ at R0 to 4.9% at R6. Lignin proportion evolution during retting is consistent with other studies

(Jankauskiene et al., 2015; Mazian et al., 2018). The increase in lignin proportion may have implications for fiber strength, and stiffness, which can influence the suitability of the fibers for different applications (Graupner, 2008). These results concerning lignin must be interpreted with some caution as other phenolic or protein components evolved during retting might be also measured through the used Klason method (Placet et al., 2017). In addition, the decrease in the proportion of hemicelluloses and pectin could lead to an increase in the relative proportion of lignin (Placet et al., 2017).

Considering our experimental conditions, it appears that biochemical variation is primarily influenced by the duration of retting. These variations in biochemical composition are probably due to microbial activities and correlate with microscopic observations that showed the decohesion of fiber bundles during retting (Bleuze et al., 2018). Fibers with a high cellulose proportion play a crucial role in providing strength

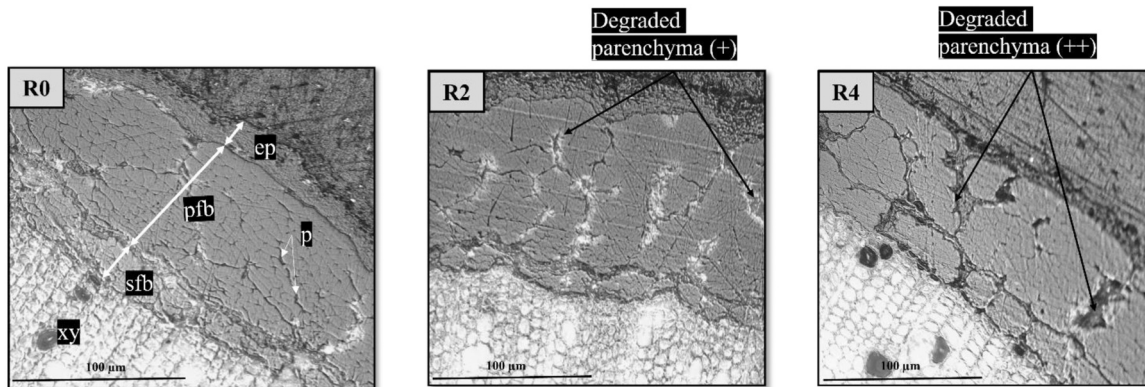


Fig. 7. Optical microscope micrographs showing cross sections of unretted hemp stem (R0), 2 weeks retted hemp stem (R2), and 4 weeks retted hemp stem (R4); (xy) xylem, (ep) epidermis, (pfb) primary fiber bundles, (sfb) secondary fiber bundles and (p) parenchyma (A). Scale bar = 100 μm.

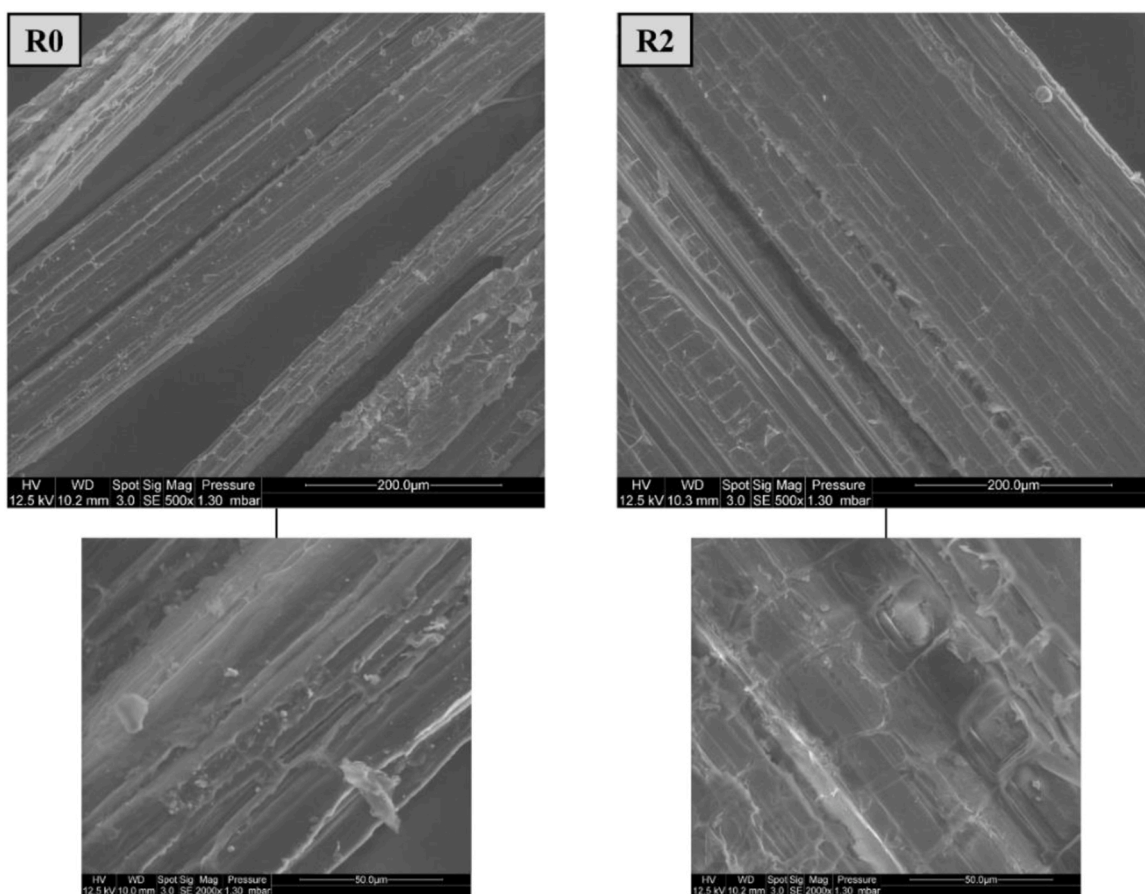


Fig. 8. SEM micrographs of unretted hemp fiber (R0) and 2 weeks retted hemp fiber (R2) (B). Scale bar = 200 μm (up) and 50 μm (down).

and stability, making them appropriate for various industrial applications. Therefore, by eliminating, lipophilic extractives, and hemicellulose, fiber properties can be enhanced (Jankauskiene et al., 2015).

3.4.2. Evolution of pectin proportion

Pectins which are part of the lipophilic extracts are mainly amorphous polysaccharides. These are the first components targeted by the activities of microorganisms during retting (Chabbert et al., 2020; Réquillé et al., 2021). Pectins are mainly found in the primary wall and the middle lamella surrounding the fibre bundles (Nykter et al., 2008). Pectin proportion decreases gradually during retting, from $7.3 \pm 0.4\%$ for unretted stems (R0) to $1 \pm 0.3\%$ after 6 weeks of retting (R6)

(Fig. 10). The most important reduction in pectin proportion (52%) is observed at the earlier stage of retting (R0 to R2). A study (Liu et al., 2015b) has shown similar results indicating that during the early stage of retting, pectins undergo rapid degradation, with the degradation rate decreasing as retting progresses. A study performed on the Bialobrzeskie hemp variety shows a similar fibers pectin proportion (1.8%) after 5 weeks of dew retting (Konczewicz et al., 2018). Another study (Mazian et al., 2018), using the same experimental method, has shown an almost 50% decrease in pectin proportion after 9 weeks of retting for the Santhica 27 variety harvested at the beginning of the flowering period, with an important degradation rate at the first stages of retting (R0-R3).

The results highlighted that retting had a major impact on the

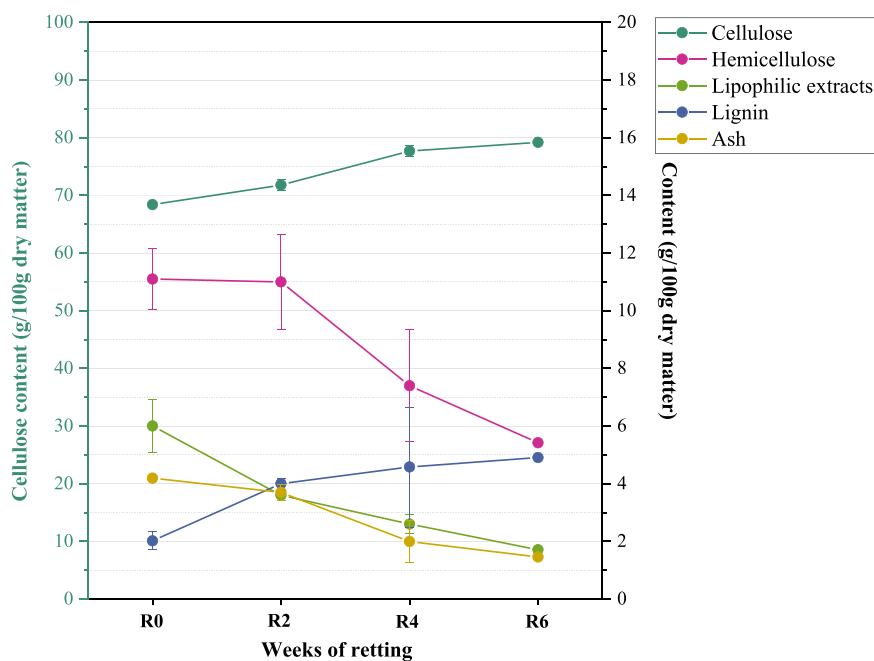


Fig. 9. Evolution of cellulose, hemicellulose, lipophilic extracts, lignin, and ash proportions during retting of unretted hemp fiber (R0) and retted hemp fiber for 2 (R2), 4 (R4), and 6 (R6) weeks. Bars represent the average of duplicate samples for each data point.

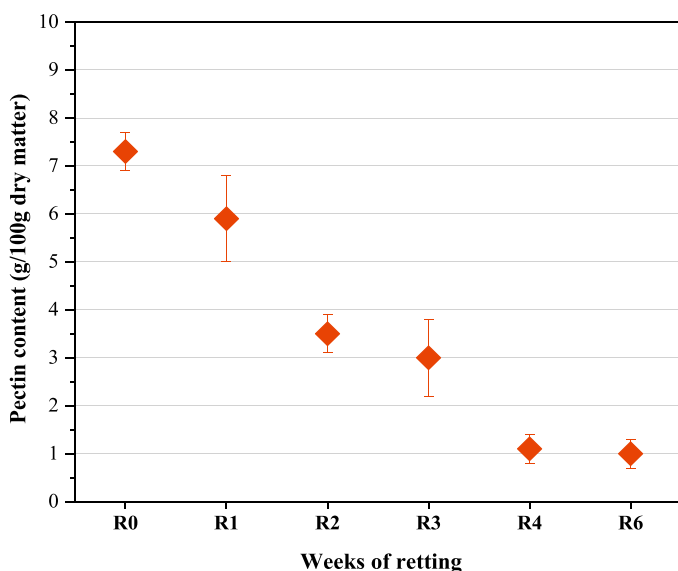


Fig. 10. Evolution of pectin proportion during retting of unretted hemp fiber (R0) and retted hemp fiber for 1 (R1), 2 (R2), 3 (R3), 4 (R4), and 6 (R6) weeks. Bars correspond to the average of five replicates.

chemical composition of the fibers, resulting in a significant loss of pectic substances. As for the hemicelluloses, pectins are considered cementitious components of hemp fiber bundles. The middle lamellae, which allow intercellular adhesion and tissue cohesion between the elementary fibers, are rich in pectin. The decrease in pectin proportion may lead to an increase in the separation of fibre bundles within the bast fibers (Réquilé et al., 2021). Thus, during the retting process, the fibre properties can be improved by removing pectin, since pectin is considered one of the main impurities in fibers that prevent any fiber from becoming spinnable to form a yarn for an end-use application (Pickering et al., 2007; Shuvo, 2020).

3.4.3. Cellulose crystallinity

Cellulose is a homogeneous linear polymer consisting of β -(1 \rightarrow 4) glycosidic chains aggregated into microfibrils, including highly crystalline parts that can resist enzymatic or chemical degradation (Thomsen et al., 2005; Morin-Crini et al., 2019). The highest percentage of crystalline cellulose is found in the cortex layer, which contains the bast fibers (both primary and secondary fiber layers) (Amarasinghe et al., 2022). Cellulose proportion and especially crystallinity are important biochemical and physicochemical parameters that have a strong impact on fiber properties (stiffness, stability, and strength) (Lee et al., 2020). For that, X-ray diffraction analyses are performed to monitor the evolution of cellulose organization of unretted and retted hemp fibers during retting. Previous observations have indicated that the removal of non-cellulosic materials (such as lignin, pectin, hemicellulose, and amorphous cellulose) may lead to an increase in both the cellulose fraction and cellulose crystallinity (Mazian et al., 2018, 2019). The presence of amorphous components between cellulose microfibrils leads to disoriented zones that can affect their crystallinity since the crystalline nature of cellulose is influenced by the packing of adjacent chains (Marrot et al., 2013).

Fig. 11 shows X-ray diffractograms of hemp fibers at different retting times (R0, R2, R4, and R6). The diffraction patterns of cellulose I in the range of 10–40° (2 θ) highlight three main reflection peaks for the crystalline phases at $2\theta \approx 14.5^\circ$ (101 diffraction plane), 16.2° (10 $\bar{1}$ diffraction plane) and 22.5° (002 diffraction plane), with the amorphous phase observed at $2\theta \approx 18.5^\circ$ (021 diffraction plane). These peaks are used to analyze the XRD spectra of the hemp fiber by determining the crystalline order index (CI) (Table 1) which is correlated to the arrangement of individual fibrils within a cellulose fiber.

Results show that CI increased slightly after two weeks of retting from $63.1 \pm 1.5\%$ for unretted fibers (R0) to $66.9 \pm 0.3\%$ for retted fibers (R2). This index remained constant until the end of retting (R6). With such a high crystallinity index ($\approx 67\%$), Futura 75 fibers can be expected to have high mechanical properties, as higher crystallinity gives the fibers higher stiffness or strength (Shuvo, 2020). Some studies (Mazian et al., 2018, 2019), using the same experimental method have shown an increase in cellulose crystallinity index of 20% and 11% respectively after 9 weeks of retting for the Santhica 27 variety

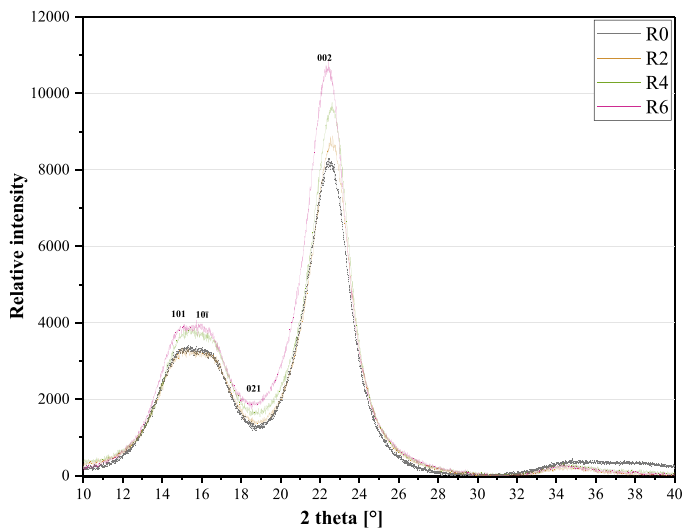


Fig. 11. Diffractogram of unretted (R0) and retted hemp fibers for 2 (R2), 4 (R4), and 6 (R6) weeks.

Table 1

Crystallinity order index of unretted (R0) and retted hemp fibers (R2, R4, and R6). The standard deviation corresponds to the average of three replicates.

Retting time	Crystallinity index
R0	63.1 ± 1.5
R2	66.9 ± 0.3
R4	67.0 ± 0.3
R6	66.8 ± 1.1

harvested at the beginning and end of the flowering periods.

During flax dew-controlled retting (19 days period), the cellulose crystallinity index measured also by XRD, shows an increase of 8% (Bourmaud et al., 2019). However, Réquillé et al. (Réquillé et al., 2020) exhibit that cellulose crystallinity remained fairly constant during

retting for the Futura 75 variety with a crystallinity index of 70% which is close to the present results.

3.5. Evolution of the hemp stems surface colonization during retting

Scanning electron microscopy (SEM) surface observations of the stems (Fig. 12) exhibit the development of microbial communities during field retting. Stems surface before retting (R0) exhibits a few trichomes. After 1 week of retting (R1), microbial communities begin to colonize the stem surface and gradually cover the entire surface after 4 weeks of retting (R4).

This evolution of the surface of hemp stems during retting has already been reported (Jankauskiene et al., 2015; Liu et al., 2017; Bleuze et al., 2018; Bleuze et al., 2020). During the fungal invasion, hyphae use natural openings on the stem surface, such as a detached trichome (Fig. 12.e). This allows hyphae to enter epidermal cells directly and colonize intact cells. The hyphae can also penetrate inside epidermal cells after the cutinase activity of soil fungi that degrade the cuticular layer. This change in the structure of the stem favors the invasion of other fungi and bacteria that can degrade the pectin of the cortical layer of stems (parenchyma cells and middle lamellae of fibers walls) (Fernando et al., 2019). In addition, the large colonization during retting observed in this study is probably due to the favorable climatic conditions (optimal temperature, high humidity, and important rainfalls (90 mm after 3 weeks of retting) which favored the development of the microbial biofilm.

3.6. Evolution of microbial communities during retting

3.6.1. DNA yield and purity

The total yields of purified genomic DNA are measured spectrophotometrically (Quant-iT™ PicoGreen™ dsDNA Assay kit). Fig. 13 shows DNA yields (µg/g of dry matter) and purity levels ($A_{260/280}$ and $A_{260/230}$) for soil and hemp stem samples as a function of the retting duration. In this study, soil samples (R0, R2, and R4) were used as a reference compared to stem samples.

For soil samples (Fig. 13.A), DNA yields increased during retting from 18.7 µg/g of dry matter (R0) to 21.7 ± 2.9 µg/g of dry matter after 4 weeks of retting (R4), with no significant difference (p -value ≥ 0.05)

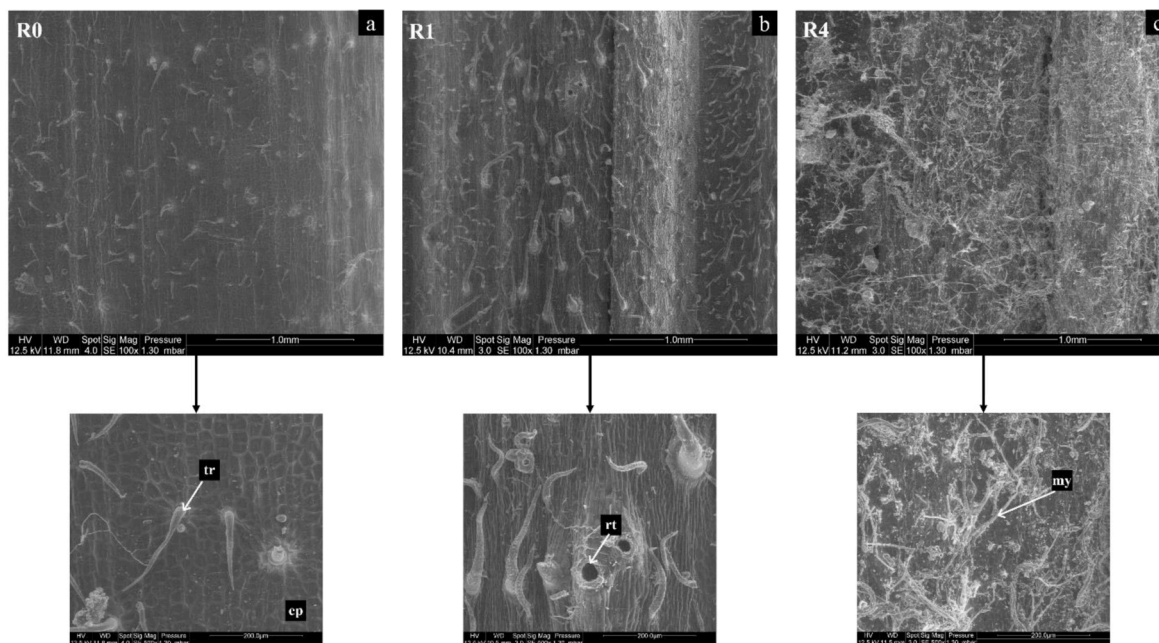


Fig. 12. SEM images of the stem surfaces during retting. a) Unretted stems (R0), b) after 1 week of retting (R1), and c) after 4 weeks of retting (R4); scale bar = 1 mm. d) epidermis (ep) and trichome (tr), e) remnant of trichome (rt), f) mycelium (my); scale bar = 200 µm.

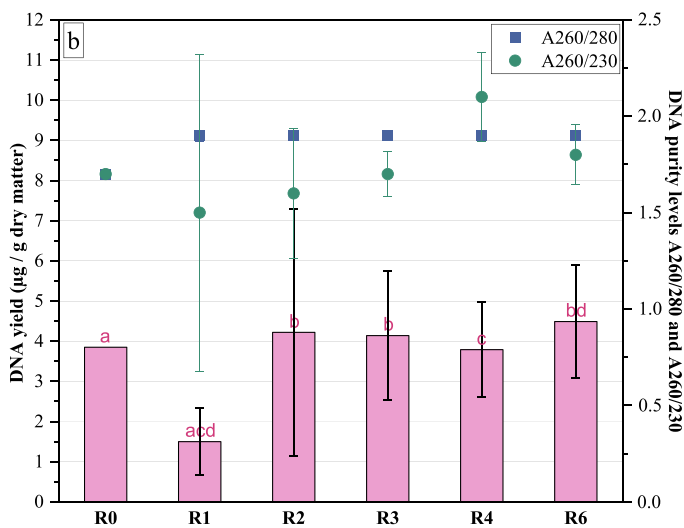
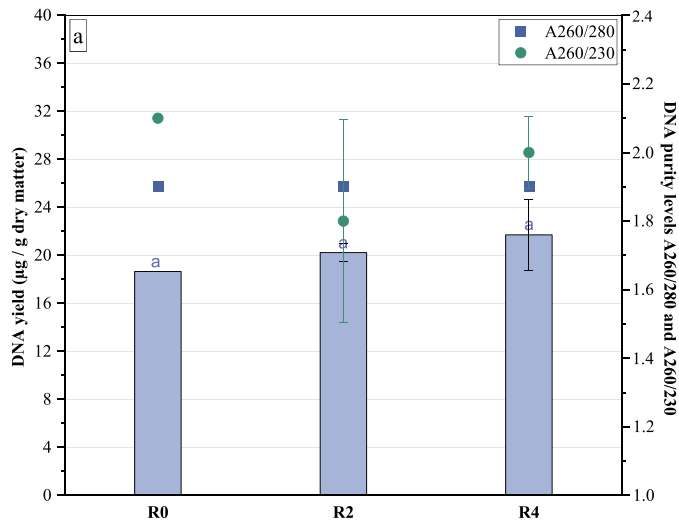


Fig. 13. Evolution of DNA yield and purity extracted from the soil at R0 and after 2 (R2) and 4 (R4) weeks of retting (A). Evolution of DNA yield and purity extracted from unretted hemp stem (R0) and retted hemp stem after 1 (R1), 2 (R2), 3 (R3), 4 (R4), and 6 (R6) weeks (B). Bars correspond to the average of five replicates (corresponding to five swaths), except for R0 (1 replicate). Letters above the bars represent significant differences between samples. Two samples sharing the same letter are not significantly different, while different letters indicate statistically significant differences (p -value < 0.05).

between the different weeks of retting (R0 vs R2; R0 vs R4 and R2 vs R4). For stem samples (Fig. 13.B), the DNA amounts increased from 3.9 µg/g of dry matter for unretted Futura 75 stems (R0) to 4.5 ± 1.4 µg/g of dry matter after 6 weeks of retting (R6). Significant differences (p -value < 0.05) are observed between the different weeks of retting (R0 vs (R2-R6); R1 vs (R2-R3), R2 vs R4, R3 vs R4, and R4 vs R6). These results suggest that the retting process has an impact on the DNA yields which depends on the sample type (soil or stem). This could reveal variations in microbial colonization during retting. Thus, an evolution of stem microbial colonization depending on the retting stage can be suggested.

DNA purity, estimated through the ratio $A_{260/280}$ and $A_{260/230}$ is used to verify the quality of extracted DNA from soil and stem samples. For soil samples, $A_{260/280}$ ratio values are within the acceptable range (1.8–2.2) and are stable at 1.9 ± 0.0 . Similar results are observed for stem samples where $A_{260/280}$ ratios are also stable at 1.9 ± 0.0 (Bou Orm et al., 2023). The $A_{260/230}$ ratio is also used as a secondary measure of DNA purity. The recommended $A_{260/230}$ ratio ranges from 2.0 to 2.2. When the ratio value is inferior to 1.5, it is assumed that contaminants

(organic compounds, proteins, or chaotropic agents) have not been removed during DNA extraction and purification steps. For soil samples, $A_{260/230}$ ratio values vary between 1.8 ± 0.3 and 2.1 ± 0.0 . For stem samples, $A_{260/230}$ ratio values vary between 1.5 ± 0.8 and 2.1 ± 0.2 . These values ($A_{260/280}$ and $A_{260/230}$) reveal that the extracted DNA can be used for further characterization of microbial communities (density, structure).

3.6.2. Bacterial and fungal community densities

To confirm the microbial invasion observed by using scanning electron microscopy, it was important to carry out quantitative measurements (microbial cell densities) to confirm qualitative microscopic observations. Therefore, during the retting process, the bacterial and fungal cell densities colonizing both stem surface and soil were estimated by qPCR (Fig. 14). A similar evolution of bacterial and fungal populations in the soil is observed throughout the retting process (Fig. 14.A). For fungal communities, the number of DNA copies / g of the

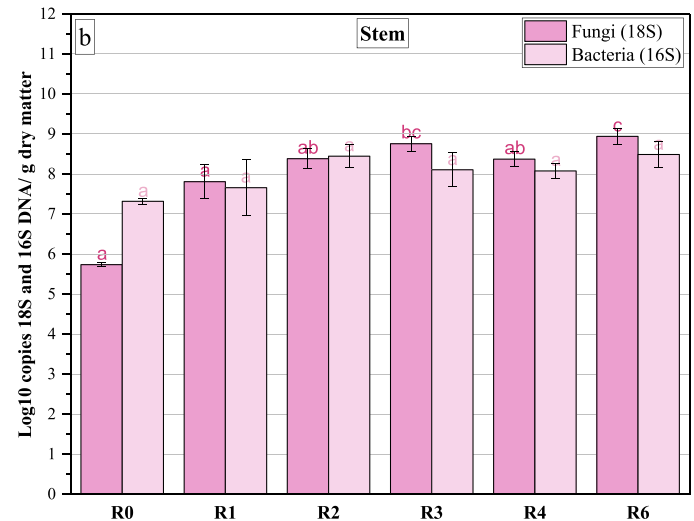
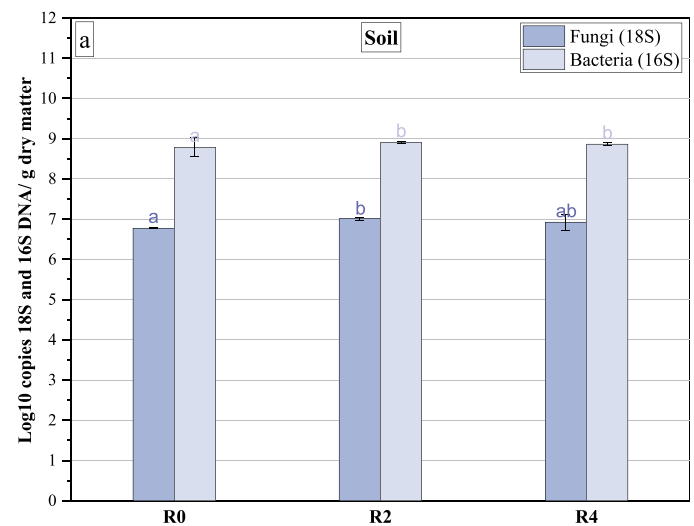


Fig. 14. Evolution of bacterial (16 S) and fungal (18 S) community densities determined from the soil at R0 and after 2 (R2) and 4 (R4) weeks of retting (A). Evolution of bacterial (16 S) and fungal (18 S) community densities determined from unretted hemp stem (R0) and retted hemp stem after 1 (R1), 2 (R2), 3 (R3), 4 (R4), and 6 (R6) weeks. Bars correspond to the average of five replicates corresponding to the studied five swaths. Letters above the bars represent significant differences between samples. Two samples sharing the same letter are not significantly different, while different letters indicate statistically significant differences (p -value < 0.05).

dry matter remains around 10^7 which is lower than that of bacterial communities (10^9 / g of dry matter). On the other hand, for stems, the density values (Fig. 14.B) remain stable after 2 weeks of retting, with similar development of the fungal and bacterial populations. An increase in cell densities (from 10^6 to 10^8 DNA copies/g of dry matter and from 10^7 to 10^8 DNA copies/g of dry matter for fungal and bacterial communities respectively) is observed.

Taken together, the microscopic observations combined with the stem color changes, biochemical analysis, and microbial cell density values provided evidence of important changes on the stem surface after 2 weeks of retting with microbial colonization. However, measuring population densities is not sufficient to understand the biological mechanisms of retting. Temporal dynamics of microbial communities can also provide deep insights for understanding the biological mechanisms that occur during retting and for the characterization of cell wall component degraders. High-throughput sequencing (targeted-metagenomics) may allow us to characterize the bacterial and fungal community organization (“who is there”) during retting (Djemiel et al., 2017; Maron et al., 2011). In addition, metaproteomics analysis can also identify enzymes involved in the degradation of plant cell wall components during retting, targeting the truly active degrading community (“who does what”) (Djemiel et al., 2022; Maron et al., 2007).

3.7. Statistical analysis

Fig. 15 shows a heatmap that represents the Spearman correlation coefficients between different variables. The heatmap is color-coded, with red color indicating positive correlations and blue color indicating negative correlations. The color intensity represents the correlation absolute values, e.g., bright red stands for high positive correlations and light blue for moderate negative ones. The variables are organized into three groups: “physicochemical parameters”, “climatic parameters” and “biological parameters”. The physicochemical parameters include variables such as pectin, crystallinity, ash, lignin, hemicellulose, cellulose, and lipophilic extracts, while the microbial parameters include variables related to the density of bacterial and fungal communities. The climatic parameters include air (named humidity, maximum and minimum temperature, and rainfall) and soil conditions (humidity named soil_hum and temperature named soil_temp). The heatmap reveals both positive and negative correlations among the variables. However, the variables that exhibited a statistically significant p-value (p -value ≤ 0.1) for a two-sided independence test have been considered.

The most important correlations are between microbial community variables, pectin proportion, and air temperature. Regarding the microbial community variables, significant negative correlations (p -value ≤ 0.1) are found between the density of fungal communities and the pectin proportion. This suggests that higher fungal density is linked to increased degradation of pectin in retted hemp fibers, resulting in an overall reduction of their proportion. This finding is consistent, as fungi can produce pectinolytic enzymes that break down the pectin components in plant cell walls (Reignault et al., 2008). In addition, a significant negative correlation (p -value ≤ 0.1) is observed between the density of fungal communities and the maximum air temperature, indicating that fungal density is negatively associated with higher temperatures. This is consistent with the accepted principle that fungal growth is favored by moderate temperatures (Pietikäinen et al., 2005).

On the other hand, a significant positive correlation (p -value ≤ 0.1) is found between the pectin proportion and both maximum and minimum air temperatures. This suggests that extreme air temperatures (maximum and minimum) may lead to higher levels of pectin proportion, thus reducing pectin degradation. This finding reinforces the link between temperature, fungal community presence, and pectin degradation observed in previous correlations, indicating that temperature fluctuations could influence fungal community density and, consequently, the rate of pectin degradation.

The correlations observed between climatic data, such as soil

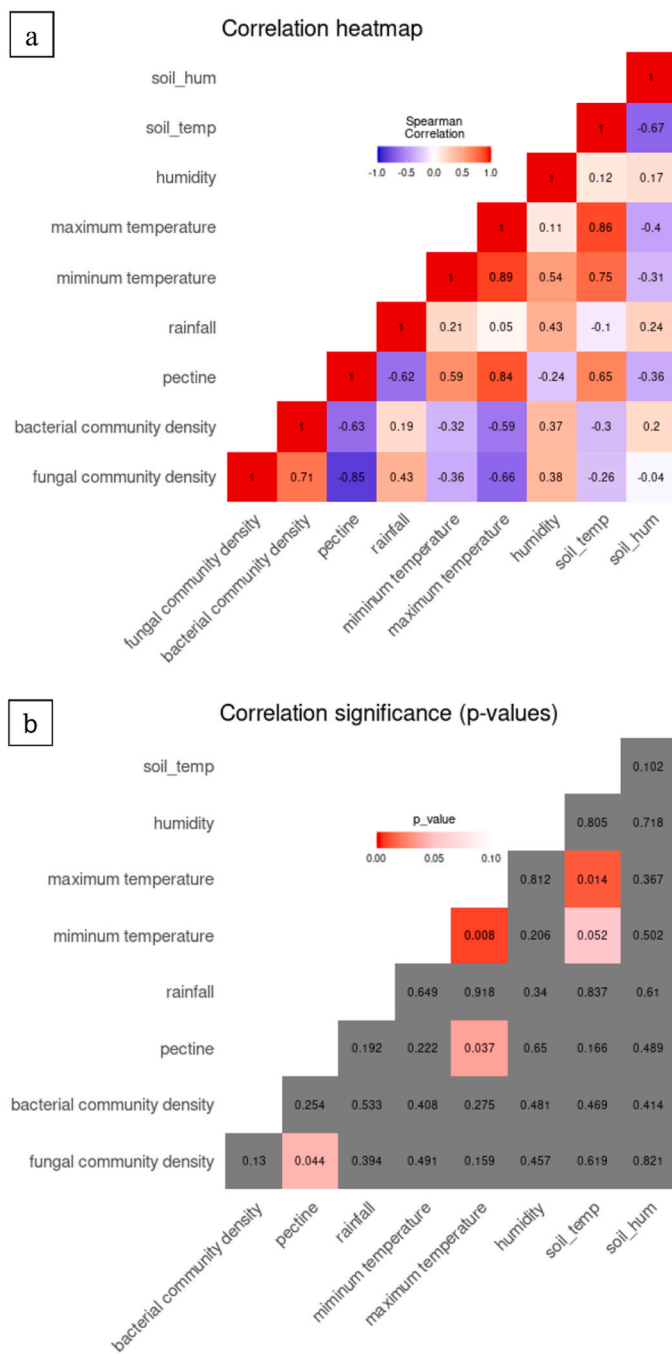


Fig. 15. Spearman’s correlation matrix heatmap (a) and correlation significance (b) obtained between climatic conditions, fiber physicochemical properties, and bacterial and fungal community density. Positive correlations are shown in red, and negative correlations are shown in blue.

temperature and air temperature suggest that the use of the data from the meteorological station can provide sufficient information without the need for additional soil sensors.

These results suggest a significant correlation between microbial community variables and climatic data during the hemp field retting exists. Some climatic conditions may influence the composition of retting microbial communities, potentially impacting the retting process and subsequently affecting the properties of the resulting hemp fibers. The use of Spearman’s correlation coefficient is important in identifying and quantifying these relationships. The findings provide important insights into the effects of environmental factors on the retting process. It is evident that these factors cannot be controlled, posing a challenge in

monitoring the retting process to produce high-quality fibers.

Recently, the study of Chabbert et al. (2023) also investigated field retting of hemp and employed statistical methods to identify the factors influencing the retting process. This study explored how factors such as hemp harvest date, weather conditions, and soil type influence the retting process. The study employs an experimental field trial with two hemp harvest scenarios and two soil types commonly found in agricultural areas. The study revealed that retting kinetics follow similar patterns regardless of soil type. They find that temperature and cumulative radiation during retting stages strongly correlate with retting kinetics, while the cumulative amounts of rain and dew have a lesser impact. In addition, the study suggests the potential development of tools to monitor changes in hemp stem quality based on retting progress.

4. Conclusion

Field retting is classically performed in northern France where the climatic conditions are favorable to implement it. However, the results of this study indicate that field retting can be also achieved successfully in a Mediterranean climate (southern France). This study revealed that after 6 weeks of retting of hemp stems harvested at the end of the flowering period, a significant change in the color of the stems and fibers from light green to black is observed, which is due to the colonization of the surface of the stem by microorganisms. Large microbial colonization has been highlighted after 4 weeks of retting (SEM images). The evolution of microbial cell densities at the hemp stem surface indicates an increase in population densities of both bacterial and fungal communities (from 10^6 to 10^8 DNA copies/g of dry matter and from 10^7 to 10^8 DNA copies/g of dry matter for fungal and bacterial communities respectively). These results confirm the importance of microbial populations in this retting process through the production of pectinolytic exoenzymes. Decohesion of the fiber bundles due to degradation of the cortical parenchyma and other cementing compounds is also observed. This result is confirmed by biochemical analysis which exhibits degradation of the non-cellulosic material (pectin, hemicellulose) with an increase in the cellulose proportion (from $68.4 \pm 0.1\%$ at R0 to 79.2% at R6) and its degree of crystallinity (from $63.1 \pm 1.5\%$ at R0 to $66.8 \pm 1.1\%$ at R6). The use of a statistical correlation approach in this study concerning hemp field retting, allowed us to explore the relations between different types of variables, to determine their statistical significance, and to provide predictive elements to improve retting. Significant correlations were observed between microbial densities and chemical components of hemp fibers, microbial densities, and climatic data, as well as between chemical components of hemp fibers and climatic data. The correlations observed suggest that the use of the data from the meteorological station can provide sufficient information without the need for additional soil sensors. Moreover, results provide important insights into the effects of environmental factors on the main actors of retting, i.e., microbial communities, and thus on the retting process.

Therefore, it is essential to supplement the present findings with a comprehensive investigation of the microbial communities involved in this process and to relate these data to the different properties of the fibers (biochemical, physicochemical, and mechanical). Furthermore, more studies are needed to understand the impact of abiotic factors (temperature, humidity, etc.) on biotic factors (microbial communities). Finally, by establishing connections between these various data, it will be possible to define simple indicators that can help determine the progression of retting.

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CRediT authorship contribution statement

Sandrine Bayle: Writing – review & editing, Supervision, Project administration, Funding acquisition. **Nicolas Sutton-Charani:** Writing – review & editing, Software. **Eliane Bou Orm:** Writing – review & editing, Writing – original draft, Visualization, Validation, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Luc Malhautier:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition, Conceptualization. **Anne Bergeret:** Writing – review & editing, Validation, Supervision, Project administration, Funding acquisition. **Jean-Charles Benezet:** Writing – review & editing, Supervision, Project administration, Funding acquisition.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data Availability

Data will be made available on request.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at [doi:10.1016/j.indcrop.2024.118487](https://doi.org/10.1016/j.indcrop.2024.118487).

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