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DISSERTATION / DOCTORAL THESIS

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„Fun with Dung: Upcycling of herbivore manure into value-added products“

verfasst von / submitted by

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„I’m going slightly mad“

-Queen

Abstract

Livestock farming for the production of animal products requires large quantities of biomass as feed. Animals are typically fed grasses, hay, corn and silage. Feed is partially digested resulting in large quantities of manure still containing intact plant fibres. Manure represents a huge biomass resource that up to now has mainly been used as farm fertiliser but is also associated with an environmental burden. Manure is one of the biggest greenhouse gas emitters within the agricultural sector and contributes to pollution of land and eutrophication of surface water. One possibility to reduce environmental concerns is the use of manure as substrate to produce biogas, which provides the additional advantage of generating an improved farm fertiliser. Anaerobic fermentation process yielding biogas – a mixture primarily comprised of methane and carbon dioxide – is incomplete and the components present in the fermentation residue are currently still underutilised. Especially for intensive farming operations, a manure management system is needed allowing to tackle environmental concerns while enabling the valorisation of manure as a raw material resource.

The aim of my work was a holistic approach to upcycle low-grade biomass, typically not used in pulp production, initially utilising herbivore digestion systems and the resulting manure into value-added products, i.e. biogas, farm fertiliser and pulp fibres. A manure management system leading to a circular material stream and full manure utilisation is proposed. Due to their high lignocellulosic fibre content, elephant and horse manure were used as model system. Manure was used as substrate for biogas production, the fermentation residue extracted and a fertilizer precursor obtained from the liquid residue. The residual lignocellulosic fibres present in the biogas residue were used for the production of pulp fibres, which were subsequently processed into nanocellulose and nanopapers. Biogas in high yields (399 and 207 $L_N\ kg^{-1}_{VS}$ for elephant and horse manure, respectively) was produced. I could show that manure and fermentation residues are a valuable resource for the isolation of nanocellulose and nanopaper production. Properties of fibres and papers were comparable to literature reporting values for nanopapers produced from high-grade biomass, such as wood. Additionally, I could show that the fermentation process enables the up-concentration of lignin on lignocellulosic fibres

resulting in nanolignocellulse with lignin contents of up to 29 wt%, which resulted in a reduced water affinity of nanopapers produced thereof.

The environmental impact of the proposed manure valorisation system was evaluated by lifecycle assessment and compared with traditional pulping processes using wood chips and a biogas production process based on agricultural waste. The newly established process was superior in all environmental impact categories.

Animal manure can also be upcycled by using it as substrate for fungal cultivation. Two species of white rot fungi were cultivated on elephant manure for different growth periods and the resulting fungal biomass modified lignocellulosic fibres extracted. It was possible to produce hierarchical composite papers taking advantage of the hybridization of nanosized chitin-β-glucan fibrils, present in the cell wall of fungi, and the micro-sized lignocellulosic fibres. Resulting papers exhibited with improved tensile properties and reduced wettability compared to papers produced using lignocellulosic fibres extracted from manure.

Zusammenfassung

Viehzucht zur Erzeugung tierischer Produkte erfordert große Mengen an Biomasse als Futtermittel. Die Tiere werden in der Regel mit Gräsern, Heu, Mais und Silage gefüttert. Dabei wird das Futter nur teilweise verdaut, so dass große Mengen an Dung entstehen, welche noch intakte Pflanzenfasern enthalten. Dadurch stellen Tierexkremente eine riesige Biomasseressource dar, die bisher vor allem als Dünger verwendet wurde, aber auch mit erheblicher Umweltbelastung in Verbindung gebracht wird. Tierexkremente sind einer der größten Verursacher von Treibhausgasemissionen in der Landwirtschaft und tragen zur Bodenbelastung und zur Eutrophierung von Oberflächengewässern bei. Eine Möglichkeit die negativen Umweltbelastung zu verringern, ist die Verwendung von Tierexkrementen als Substrat zur Biogasherstellung. Dies bietet den zusätzlich Vorteil der Erzeugung eines Wirtschaftsdünger mit erhöhter Nährstoffverfügbarkeit. Der anaerobe Gärungsprozess, bei dem Biogas, ein Gemisch aus Methan und Kohlendioxid, entsteht, ist unvollständig, und die Bestandteile des Gärrestes werden derzeit noch nicht optimal genutzt. In Anbetracht der Intensivierung landwirtschaftlicher Produktion gewinnen Aufwertungssysteme, welche es ermöglichen die damit assozierten negativen Umwelteinflüsse zu minimieren und gleichzeitig unvermeidlich anfallenden Dung als Rohstoffressource zu nutzen, immer mehr an Bedeutung. Ziel meiner Arbeit war ein ganzheitlicher Ansatz zur Verwertung von Biomasse niedriger Qualität, die in der Regel nicht für die Celluloseproduktion herangezogen wird, durch die Nutzung von Verdauungssystemen der Herbivore und Transformation der daraus resultierenden Tierexkrementen zu Produkten mit höherer Wertschätzung: Biogas, Wirtschaftsdünger und Cellulosefasern. Es wird ein Managementsystem vorgeschlagen, das zu einem zirkulären Materialfluss und einer vollständigen Verwertung der Tierexkremente führt. Aufgrund ihres hohen Gehalts an Lignocellulosefasern wurden Elefanten- und Pferdemist als Modellsystem verwendet. Der Dung wurde zunächst als Substrat für die Biogaserzeugung verwendet, der feste Gärrückstand extrahiert und aus dem flüssigen Rückstand ein Düngemittelvorprodukt gewonnen. Aus den im Biogasrückstand enthaltenen lignocellulosehaltigen Fasern wurden Cellulosefasern isoliert, die anschließend zu

Nanocellulose und Nanopapieren verarbeitet wurden. Es wurde Biogas in hoher Ausbeute (399 und 207 $L_N\ kg^{-1}_{vs}$ Elefanten- bzw. Pferdemist) hergestellt. Ich konnte zeigen, dass sowohl Dung, als auch Gärreste eine wertvolle Ressource für die Produktion von Nanocellulose und die Herstellung von Nanopapieren ist, welche vergleichbare Eigenschaften mit äquivalenten Materialien, die aus hochwertiger Biomasse (z.B. Holz) gewonnenen wurden, aufweisen. Darüber hinaus konnte ich zeigen, dass der Fermentationsprozess die Anreicherung von Lignin auf Lignocellulosefasern ermöglicht, wodurch Nanocellulose mit einem Ligningehalt von bis zu 29 Gew.-% hergestellt werden konnte, welche eine geringere Wasseraffinität der daraus hergestellten Nanopapiere bedingte.

Die Ökobilanz des vorgeschlagenen Systems wurde untersucht und mit einem konventionellen Celluloseherstellungprozess unter Verwendung von Holzspänen und der Erzeugung von Biogas aus Landwirtschaftsabfällen verglichen. Das neu etablierte Verfahren war in allen Umweltwirkungsindikatoren überlegen.

Tierische Exkremente können ebenfalls als Substrat für die Pilzzucht verwendet werden. Zwei Arten von Weißfäulepilzen wurden auf Elefantendung für unterschiedliche Wachstumszeiten kultiviert und die daraus resultierenden durch Pilzbiomasse modifizierte Lignocellulosefasern extrahiert. Davon konnten hierarchische Papiere hergestellt werden, die die Vorteile der nano-dimensionalen Chitin-β-Glucanfibrillen, die in der Zellwand der Pilze vorkommen, und der mikrometergroßen Lignocellulosefasern nutzen. Diese Papieren hatten verbesserte Zugeigenschaften und geringerer Benetzbarkeit, im Vergleich zu Papieren, die aus Elefantendung extrahierten Lignocellulosefasern hergestellt wurden.

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List of Scientific Publications

Publication I

Weiland, K., Wlcek, B., Krexner, T., Kral, I., Kontturi, E., Mautner, A., Bauer, A., and Bismarck, A., 2021, 'Excellence in Excrements: Upcycling of herbivore manure into nanocellulose and biogas ', *ACS Sustainable Chemistry and Engineering*, 9, 46, 15506–15513.

KW performed all experimental work, performed all data analysis, except for biogas experiments and wrote the manuscript. BW performed biogas experiments and analysis. TK contributed to chemical analysis. IK contributed to conceptualisation and revision of the draft. EK contributed to manuscript preparation. AM supervised the work and revised the draft. ABa and ABi conceptualised and supervised experiments and revised the draft. All authors contributed to manuscript review and editing.

Publication II

Weiland, K., Alge, K., Mautner, A., Bauer, A., and Bismarck, A., 2022, 'Horsepower: Horse manure as resources for biogas and nanolignocellulosic fibres', submitted 2022

KW performed all experimental work, performed all data analysis and wrote the manuscript. KA contributed to biogas experiments and analysis. AM supervised the work and revised the draft, ABa and ABi conceptualised the experiments, supervised and revised the draft. All authors contributed to manuscript review and editing.

Publication III

Weiland, K., Jones, M., Zinsser, F., Kontturi, E., Mautner, A., Bismarck, A., 2021. 'Grow it yourself composites: delignification and hybridisation of lignocellulosic material using animals and fungi', *Green Chemistry*, 23, 7506-7514.

KW performed all experimental work, performed all data analysis and wrote the manuscript. MJ contributed to experimental design, sample preparation and manuscript preparation. FZ contributed to sample preparation. EK performed sugar analysis experiments and contributed to manuscript preparation. AM contributed to manuscript revision and supervision. ABi

conceptualised and supervised the work. All authors contributed to manuscript review and editing.

Publication IV

Krexner, T., Bauer, A., Zollitsch, W., **Weiland, K.**, Bismarck, A., Mautner, A., Medel-Jiménez, F., Gronauer, A., Kral, I., 2022 'Environmental life cycle assessment of nano-cellulose and biogas production from manure', *Journal of Environmental Management*, 314,115093.
TK modelled and performed LCA and wrote manuscript. KW provided all data. IK contributed to methodology, wrote manuscript and supervised worked. AG and AB supervised the work. Aba, IK and AB conceptualised the work. All authors contributed to manuscript review and editing.

Publications not included in this thesis

Mautner, A., Kwaw, Y., **Weiland, K.**, Mvubu, M., Botha, A., Jacob John, M., Mtibe, A., Siqueira, G., Bismarck, A., 2019 'Natural fibre-nanocellulose composite filters for the removal of heavy metal ions from water' *Industrial Crops and Products*, 133, 325-332,

Jones, M, **Weiland, K**, Kujundzic, M, Mautner, A, Bismarck, A & John, S 2019. 'Sustainable Mycelium-derived Chitinous Thin Films'. In 22nd International Conference on Composite Materials, Melbourne, Australia.

Weiland, K, Jones, M, Fortea-Verdejo, M, Mautner, A, Bismarck, A & John, S 2019. 'Waste-derived Nanocellulose-chitin Hybrid Materials with Tunable Physical Properties'. In 22nd International Conference on Composite Materials, Melbourne, Australia.

Jones, M, **Weiland, K**, Kujundzic, M, Mautner, A, Bismarck, A & John, S 2019. 'Chitin-chitosan Thin Films from Microbiologically Upcycled Agricultural By-products'. In 13th International Conference on the Mechanical Behaviour of Materials, Melbourne, Australia.

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List of Abbreviations and Symbols

Symbol	Parameter	Unit
A	Area	[m^2]
E	Elastic modulus	[GPa]
ε	Strain to failure	[%]
G	Grammage	[g m^{-2}]
m	Mass	[g]
ρ_e	Envelope density	[g cm^{-3}]
ρs	Skeletal density	[g cm^{-3}]
σ	Tensile strength	[MPa]
Φ	Porosity	[%]
W_{H20}	Water retention	[wt%]
Abbreviations		
ADF	Acid detergent fibre	
ADL	Acid detergent lignin	
AIL	Acid insoluble lignin	
Cel	Cellulose	
DM	Dry mass	
eq	Equation	
FEP	Freshwater eutrophication potential	
FRS	Fossil resource scarcity	
G	Grammage	
GHG	Greenhouse gases	
GWP100	Global warming potential	
HCel	Hemicellulose	
HCTP	Human carcinogenic toxicity potential	
HNCTP	Human non-carcinogenic toxicity potential	
LCA	Lifecycle assessment	
NDF	Neutral detergent fibre	
SEM	Scanning electron microscopy	
TAP	Terrestrial acidification potential	
TEMPO	2,2,6,6-tetramethylpiperidine-1-oxyl	
TEP	Terrestrial ecotoxicity potential	
VS	Volatile solid content	

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1 Introduction

1.1 From linear to circular: agricultural waste management in future economy

To address the challenges of a growing population and to counteract the irreversible exploitation of earth's resources a circular food economy with high efficiency should be promoted, prioritising secure food supply. Linear economic systems follow the idea of production, consumption and disposal of materials resulting in environmental damage and loss of resources. On the other hand, circular economies aim at the prevention of waste and to maintain a material's value at any point in its life, including the consideration of recycling and upcycling of waste materials generated.[1] The global economy is estimated to be less than 10% circular, with waste being mainly disposed of in landfill without any options for re- or upcycling.[2] Especially in the food production system a circular economy and a circular waste management of generated waste biomass could provide the opportunity to secure the food supply while supporting a sustainable future.[3] This could be a promising concept helping to reduce the exploitation of natural resources and to generate a self-sustaining closed loop system. The main challenges of agricultural residues being converted into biomaterials are mainly related to energy consumption, degradation processes, complexity and variability in chemical composition, presence of contaminants and social perception. Currently, waste management strategies mainly focus on a single final product valorisation and production chains have been optimised in terms of product diversification and functionality, energy or water use.[4] Focusing on all benefits that a circular waste management offers, new concepts for agricultural residues and biomass waste must be developed to be able to achieve a transdisciplinary treatment concept with multiple output systems.

1.2 Manure as waste material?

The global agricultural and food industries face the critical challenge of feeding a growing world population. Over the past decades this challenge was addressed by specification and intensification of the food production chain as well as optimisation of farm inputs, such as fertilizer application or animal feed composition.[5] Within the past 25 years the total number of

farms decreased while the number of livestock per farm increased.[6] Intensified operations, separating crop and livestock production with a large number of animals concentrated in small areas, do not only lead to increased output of the desired products but also to an increased waste production and the concentration of this waste in smaller areas. Especially the separation of animal and crop production resulted in a regression of the utilisation of waste streams.[7] For the period from 2016 to 2019 it was estimated that 1.4 billion tons of animal faeces were generated in the EU alone.[8] Thus, for livestock farming, manure is attributed to be one of the main contributors to negative environmental impact. 9.3 billion tons of CO_2 equivalent of greenhouse gases (GHG) were emitted by the agricultural sector in 2018, of which crop and livestock activities were responsible for more than half of it.[9] Manure is known to be one of the main emitters of non-CO_2 GHG within agriculture, contributing to air pollution by releasing methane, ammonia, nitrous oxides and volatile organic compounds.[10] Inadequate storage leading to run off causes eutrophication and contamination of soil and surface water by accumulation of nitrates and phosphorus.[11] In Europe the pollution costs associated with manure (mis)management resulting in such emissions are estimated to amount to 12.3 billion €/year.[12]

Manure is usually used as farm fertilizer applied by spreading on fields, substrate for composting,[13] combustion[14] and anaerobic digestion (AD),[15] which allows to recover nutrients and energy. These processes either only shift the environmental concerns to the next user or do not convert the biomass to the full extent. A solution to elevate surplus manure to a completely new level as a resource for value-added materials must be found not only in terms of valorisation of waste biomass but also in order to mitigate the environmental impact of livestock production.

To achieve this, already established manure treatment technologies could be combined with separation pathways to create a circular system for materials and energy generation. As such, manure is not only spread on fields but recycled and upcycled into multiple valuable products, such as biogas, pulp fibres and fertilizer, thus adding economic value to animal manure. Animals either graze on meadows or fields collecting the biomass required for their livelihood

or are being fed in stables, which provides another opportunity to upcycle unconventional fibre sources for pulp production. Please note it was not the aim of this study to collect manure from free roaming animals dropped on the fields but to utilise manure produced in concentrated operations in stables and/or milking parlours.

1.3 Aims and Objectives

There is sufficient evidence that certain animal manure contains valuable fibres suitable for paper production.[16-18] Thus, my hypothesis was that biomass, such as grasses, shrubs etc. serving as animal feed, being processed by the animals themselves through comminution and the action of enzymes and acid, ending up in manure will serve as ideal substrate for either biogas or fungal farming and subsequent fibre production. It was hypothesised that manure and biogas fermentation residue allow for the easy production of high-quality pulp fibres that can be subsequently processed into nanofibrillated ligno/cellulose (**Figure 1**) requiring less energy as compared to common wood pulp.

The overall aim of my thesis is to upcycle agricultural waste, in particular manure, into products, which could serve to add value to a manure waste management system leading to a circular material stream and full manure utilisation. To achieve the aim of my research the following objectives had to be addressed:

1. To assess the quality of fibres extracted directly from elephant and horse manure used as model substrates,
2. To quantify the impact of subsequent biogas generation on the fibre production from the fermentation residue,
3. To investigate the composition of all process side streams and suggest possible uses for instance as liquid farm fertilizer,
4. To demonstrate the utilisation of manure as substrate for cultivation of fungi and with simultaneous end-of-life use for the spent substrate as source for pulp and paper production,
5. To model the lifecycle (assessment) (LCA) of a future waste management system utilising farmed animal manure to its full potential for biogas and nanofibril production.

1.4 Thesis outline

Following the introduction, aims and objectives I will provide a literature review summarising the state of the art on lignocellulosic material with a special focus on nanocellulose, current manure management systems, and also biopulping using fungi is discussed. Afterwards I will briefly summarise the materials and methods and provide an executive summary and discussion of the key findings reported in my research papers.

In **Publication I** I investigated a closed loop manure management system using elephant manure as model system (**Figure 1**). Biogas from elephant manure was produced and the raw material and fermentation residue were characterised. A suitable chemical separation process was investigated to obtain and produce nanocellulosic fibres. The properties of nanocellulosic papers were studied to investigate the influence of anaerobic digestion time on fibre quality (Objective 1, 2 and 3).

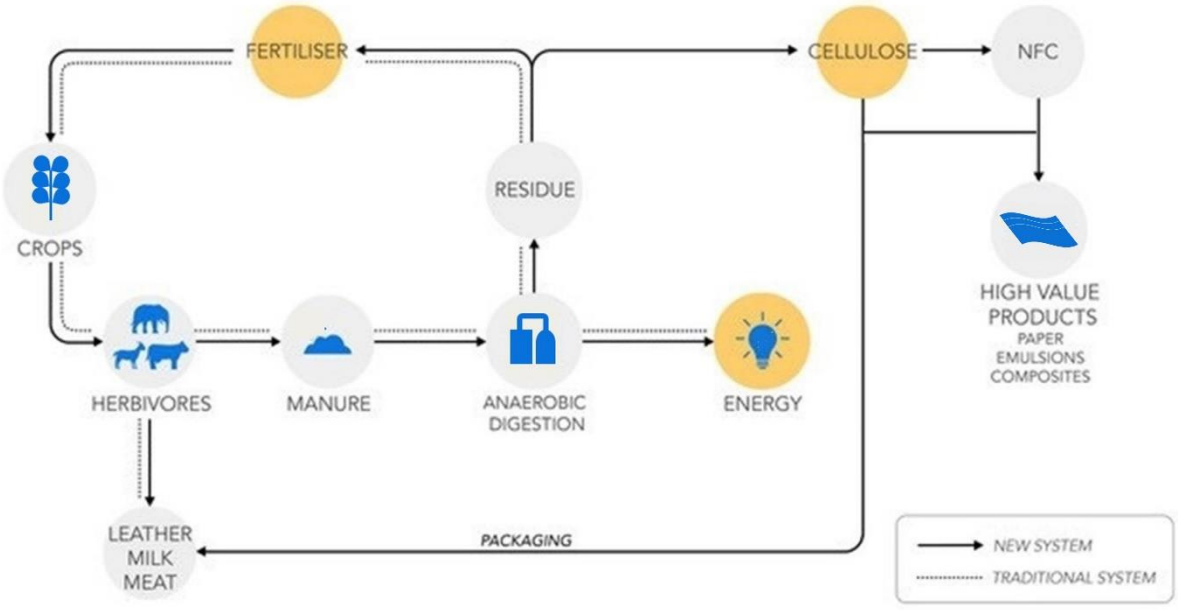

Figure 1 Proposed manure management model (Publication I) using herbivore manure for the production of biogas and the fermentation residue for the production of fertiliser precursor and cellulose for the production of NFC.[19] (Link in: accessed 24.10.22, open access under the terms and conditions of the Creative Commons Attribution (CC BY) license https://doi.org/10.1021/acssuschemeng.1c05175)

The process developed in **Publication I** and shown in Figure 1 was also applied to horse manure as raw material for validation and aiming to up-concentrate lignin on cellulose fibres during anaerobic digestion (**Publication II**) (Objective 1,2 and 3).

In **Publication III** an alternative delignification process for manure as substrate using fungal biomass to simultaneously produce composites from lignocellulosic material and the chitin-β-glucan complex (Objective 4).

In a **Publication IV** resulting from a collaboration effort with colleagues from the Institute of Agricultural Engineering of the University of Natural Resources and Life Science (BOKU) we assessed the environmental impact and lifecycle of our process (Figure 1) compared to a conventional pulping process with subsequent nanofibre production (Objective 5).

The executive summary is followed by an overall conclusion and future perspectives and suggestions for future work.

2 Literature Review

2.1 Lignocellulosic materials

Herbivores eat plant biomass, which mainly comprises three biopolymers (**Figure 2**). Cellulose, hemicellulose and lignin are combined with small fractions of extractives, proteins, inorganic compounds (typically quantified as ash content) and pectins. These components are present in different ratios, depending on plant species, development stage and can even vary within a plant. To separate the individual components, the biomass is usually treated mechanically, chemically, using acids, alkaline media or organic solvents, or aiming for a more sustainable pathway, enzymatically.[20, 21]

The main component of plant fibres is cellulose and due to its importance will be discussed in a separate section. Hemicellulose is the compatibiliser between the reinforcing component cellulose and lignin matrix and is constituted of branched polysaccharides formed by various sugars, such as glucose, xylose, mannose, galactose, arabinose, fucose, glucuronic acid and galacturonic acid. The fraction of each sugar is dependent on the source and chemical constitution of the biomass. The structure of hemicellulose is characterised by β-1, 4-linked polysaccharide backbones of glucose, mannose, or xylose.[22] Hemicelluloses are connected to cellulose via hydrogen bonds and lignin via aromatic ester bonds, thus acting as glue between cellulose and lignin and other phenolic components.[23]

Lignin, on the other hand, is a polymer network, consisting of three aromatic base monomers: coniferyl alcohol, sinapyl alcohol and p-coumaryl alcohol that are cross-linked. Lignin's specific function in plants enables water transport over long distances for particular in trees, due to its hydrophobic character and is also playing an important role in plant's resistance against insect pests.[24, 25] The pulp and paper industry is among the largest producers and providers of lignin. Approx. 100 million tonnes of lignin are produced annually, which are usually incinerated to produce energy for pulping mills.[26] A growing interest in the utilisation of lignin reflected in the and increasing number of research papers reporting multiple sustainable materials such as

nanoparticles[27], vanillin[26], epoxy resins[28] or wood based adhesives[29] using lignin as raw material.

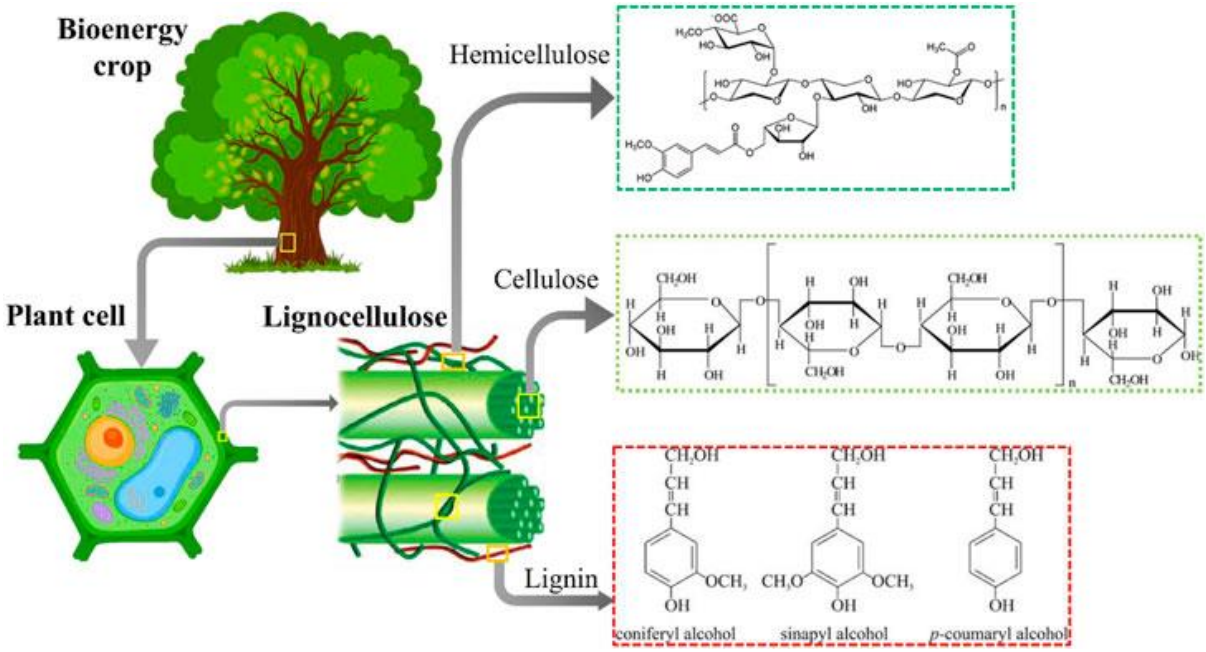

Figure 2 Structure of lignocellulosic material[30] (Link in: https://doi.org/10.3389/fnano.2021.793528, accessed 17.08.22, open access under the terms and conditions of the Creative Commons Attribution (CC BY) license)

2.1.1 Cellulose

Cellulose is the main structural biopolymer in green plants and provides mechanical stability. Wood, straw or bagasse contain approx. 40-50 wt% cellulose. Cotton seeds contain even up to 90 wt% cellulose and are an efficient source for cellulose fibers.[31] However, not only the content but also the characteristics of cellulose vary depending on the source but also on the extraction process. First described in 1839,[32] cellulose was a common raw material for the production of polymer materials before being often replaced by petrol-based polymers. Cellulose has been and is still used for production of paper,[33, 34] cardboard,[35] pharmaceutics[36, 37] and food additives.[38] It is a linear semi-crystalline polysaccharide comprising of β-1,4-connected D-glucose units.[39] Due to the high number of available –OH groups cellulose exhibits an extended hydrogen bond network.[40] The native polymer chains in green plants form elementary fibrils of 1.3 to 3.5 nm diameter via intermolecular hydrogen bonds and van der Waals forces. Elementary fibrils aggregate to nanofibrils (10 to 30 nm) and microfibres.[41] The discovery of the hierarchical structure of cellulose prompted the production of cellulosic nano

and microfibres with their associated characteristic properties. Especially nanofibrillated cellulose (NFC) has gained tremendous interest, not only in academia but also in industry due to its remarkable properties. NFC can be produced with diameters ranging from 5 to 100 nm using various top-down methods, i.e. intensive defibrillation processes such as grinding or high-pressure homogenisation,[42] that require a large energy input. The cost associated with the energy input and the high water content of the resulting NFC pulp are currently the main obstacle limiting NFC implementation into large-scale industrial production and application. *Ang et al.* reported specific energy requirements of up to 7.4 kWh kg^{-1} unrefined eucalyptus kraft pulp fibre for production of NFC using a homogenizer.[43] High quality NFC were produced by passing various pulps through a disc mill (Masuko grinder) resulting in an energy consumption of 5 and 7 kWh kg^{-1} depending on the pulp source.[44] Other reports indicate energy requirements for refining of pulp into NFC of up to 30 kWh kg^{-1}.[45-47] The introduction of various pre-treatment processes prior to mechanical grinding is highlighted as one possibility to reduce the energy consumption, for example oxidation using 2,2,6,6-tetramethylpiperidine-1-oxyl radical (TEMPO)[48], enzymatic[49] or mild acidic pre-treatments.[50] Each of these pre-treatments affects the quality (dimension, surface area, crystallinity)[51, 52] and surface character (charge content)[53] of the resulting NFC.[54]

Over the past decades the unique properties of nanocellulose have been extensively studied[55] and NFC was explored for a number of possible applications, such as hydrogels, aerogels, membranes,[56, 57] barrier coatings and as reinforcement for polymers.[58, 59] Papers produced from NFC, also called nanopapers[60], have been studied extensively.[61] Tensile properties of nanopapers produced from NFC extracted from agricultural waste streams were similar to those produced from high grade biomass, such as kraft pulp. Tensile strengths, elastic moduli and strains to failure of nanopapers made from kraft pulp were reported to range from 95 to 230 MPa, 9 to 13 GPa and 2 to 7%, respectively.[44, 62-64] NFC paper from cellulose extracted from pinecone biomass were reported to have a tensile strength of 273 MPa and an elastic modulus of 17 GPa.[65] Nanopapers made from NFC produced from lower grade biomass, such as corn cobs and maize stalks had tensile strengths in the range of 96 to 125 MPa.[66, 67] In the

pulp and paper industry wood is the main source for cellulose production, however, for nanocellulose production alternative raw materials have come into focus of research. Almost every agricultural waste or side stream, e.g. almond or peanut shells,[68, 69] corn stalk[70] or sugarcane bagasse[33, 71] but also waste from food production such as carrots[72] or sugar beet[73] were also investigated for production of NFC and nanopapers.

2.1.2 Manure as a source of cellulose

Despite multiple agricultural wastes had been explored for the production of (nano)cellulose manure is commonly not in the spotlight. Manure as a source of cellulose has been discussed mostly in the context of touristic souvenirs, for example using elephant manure mixed with recycled paper for stationery or cards as source . Using elephant manure in national parks and elephant conservation centers should draw attention to the declining numbers of these animals worldwide.[74] The extracted cellulose and produced papers had properties similar to cellulose extracted from high grade biomass, such as wood. For example, papers produced from elephant manure using an anthraquinone bleaching treatment had a tensile index of 53 N mg^{-1},[16] which is comparable to mechanical properties of copy paper.[75] Furthermore, cow manure and fermentation residue was investigated as a source for lignocellulosic fibre production demonstrating the availability of lignocellulosic fibres in manure.[76] Unfortunately, not much detail was provided in this study. Moreover, it was shown to be possible to further process the fibres extracted from cow manure to NFC.[77] Yields of cellulose ranged from 6 to 13%, which is significantly lower than compared to wood based cellulose (40-50%).[78] The low cellulose yield is due to the efficient biomass utilisation by cow's regurgitation and digestive track. This unconventional utilisation method of animal manure could potentially offer a route to the management of surplus manure and promote the development of sustainable bio-based materials.

2.2 Manure management systems

Up to now, the utilisation of manure as source of cellulose is not covered in the commonly used waste management systems. In the EU manure management is not coordinated by a single

regulation or directive but is regulated by the laws of each member state. The member states are however obliged by EU regulation (EU Nitrates Directive 91/676/EEC and Air Quality and National Emission Ceilings Directive 2016/2284) to implement manure storage measures and good agricultural practices concerning timing and amount of manure application to agricultural land.[79, 80]

To be able to manage manure and enable a circular system converting manure into value added products the composition of the raw material is crucial. The primary factors affecting the composition of manure are livestock species, feed, stable bedding and manure handling.[81, 82] Herbivores utilise lignocellulosic biomass, such as grass, hay, silage and shrubs for energy they need for living. During feed digestion, mechanical, acidic and enzymatic action, the feed is disaggregated and ultimately converted to energy, however not all fibrous material is converted and the extent of residual fibrous material depends on the digestion capability of the animal species. For instance, the four compartments in the stomach of ruminants, such as cattle and sheep, enables them to digest large amounts of lignocellulosic biomass, significantly decreasing the fibre content in corresponding manure.[83] Sheep manure contains only up to 15 wt% of cellulose fibres, while cattle manure already contains up to 27 wt% (Table 1). Monogasters such as horses and elephants, possess a simpler intestinal structure, with only one stomach and no enzymes being present to effectively break down cellulose and forages.[83] Large monogasters, including elephants, are only capable of digesting 30-40% of their feed and therefore the excreted manure can contain up to 70 wt.% fibrous material of which up to 45 wt% is cellulose (Table 1).[17] Besides undigested fibrous material nutrients, based on phosphorus and nitrogen are also present in manure.[84] These compounds originate from the residual plant material but also keratinized tissue and bacteria present in the digestive track (microbiome).[85]

Table 1 Composition (Cellulose, hemicellulose, lignin and nitrogen) of manure from different animals (cattle, horse, elephant and sheep).

	Cellulose [wt%]	Hemicellulose [wt%]	Lignin [wt%]	Nitrogen [wt%]	References
Cattle	12-27	8-21	11-52	1-3	86-90
Horse	29-38	4-28	9-10	0.2-1.7	81, 91, 92
Elephant	36-45	10-12	22-25	0.7-1.5	17, 19
Sheep	12-15	11-13	1-7	2.9	93, 94

Manure management consists of multiple processes including collection, storage, treatment, transportation and utilisation. Traditionally, manure is used in land application as farm fertilizer. Over the past decades the restrictions on land application have been tightened due to environmental concerns. Additionally, the availability of cheap synthetic fertilizer was preferred and outperformed manure due to the high transportation costs of large volumes of manure.[95] The simplest alternative way to treat solid manure is composting. It is a biooxidative process in which stabilised biomass is produced (compost). Microbes convert the substrate into compost and CO_2, NH_3, organic acids and heat resulting in volume reduction and pathogen removal.[96] This allows for safer and cheaper transportation and storage as well as facilitates spreading.[97] Composting is of certain economic interest since the process reduces the pathogen load and is beneficial to maintain or improve soil quality.[98] While compositing also takes place naturally, in order to obtain fertilizer of high quality, parameters such as particle size, nutrient content, C:N ratio and temperature have to be controlled.[99] To control parameters and guarantee constant chemical composition, bulking agents, such as straw or hay, have to be added.[100] Nevertheless, the costs of composting are considerably higher than the direct use of manure as farm fertilizer and only shift the environmental concerns as greenhouse gases are still emitted and nutrient run offs into the environment have to be prevented.

One possibility to upcycle manure into value-added products is anaerobic digestion (AD) resulting in the production of biogas. It is a key process in circular waste management because it addresses multiple challenges such as waste management, energy production, food production and nutrient recycling.[101] In 2015 in the EU 17,400 biogas plants produced 127 TJ of heat and 61 TWh of electricity.[102] During AD microorganisms convert organic material into methane, carbon dioxide and into minor amounts of other gases such as nitrogen, hydrogen

or hydrogen sulphide in an oxygen free environment. Four (sometimes three)[95] different steps can be identified: (i) hydrolysis, (ii) acidogenesis, (iii) acetogenesis and (iv) methanogenesis in which different types of bacteria degrade the substrate (Figure 5).[84] During the first step undissolved macromolecules are hydrolysed into their respective monomers. Subsequently, these monomers are converted into smaller volatile fatty acids or alcohols (e.g. methanol) by the fermenting bacteria. These compounds are then transformed into acetates, hydrogen and carbon dioxide. Methanogenic bacteria convert the residual molecules into methane and carbon dioxide.[103]

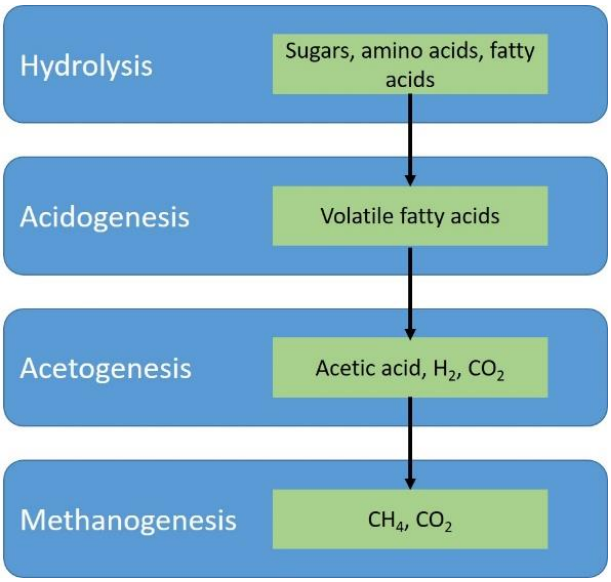

Figure 3 Different stages of biogas production and the resulting components at each stage.

During AD the pathogens removal occurs naturally, which is especially important when utilising manure as substrate considering further utilisation of the fermentation residue.[104, 105] Furthermore, only minimal odour emission has to be accepted due to decomposition of volatile compounds and the fermentation residue is an improved fertiliser in terms of availability of the nutrients for plants.[106] Taking into account the economic viability of the process, the methane yield is the main criteria. It is not only dependent on process parameters, such as pH, temperature and microbial consortium, but also on the substrate composition and, therefore, on the manure type. The C:N ratio indicates the availability of biomass convertible into methane, especially cellulose and hemicellulose content, but also nitrogen present in proteins and dead animal cells does influence the yield and composition of the biogas. The volatile solid

content is an indicator of the amount of organic material that can be converted into biogas, whereas the C:N ratio affects the system's stability. A too high nitrogen content can result in ammonia intoxication, which inhibits biogas production. Thus, co-digestion of manure substrates with other agricultural or municipal waste is commonly executed in order to adjust the constituent's ratio and compensate for the carbon deficiency in manure.[95] AD of manure of ruminants is very useful to start the fermentation process due to the high bacteria load but results usually in low methane yields, because of its high lignin content, which is not converted during biogas production.[107]

Although the main goal of anaerobic digestion is the effective conversion of lignocellulosic biomass to generate biogas in high yields, this process does not transform the entire biomass.[108] The fermentation residue comprises a significant amount of fibrous materials, such as undigested plant fibres consisting of cellulose, hemicellulose and lignin. For instance, the fermentation residue recovered after 40 days of AD of corn stover was reported to be composed of approx. 5 wt% hemicellulose, 10 wt% cellulose and 10 wt% lignin.[108] When using wheat straw as AD substrate, the residue contained 23 wt% cellulose, 15 wt% of lignin and 16 wt% hemicellulose after 45 days.[109] The fermentation residue also contains significant amounts of nutrients, such as nitrogen, phosphorus and potassium,[110] which renders it a useful fertilizer. The residue was shown to be beneficial for the physicochemical properties of soils because these minerals are easily available for plants.[111] Moreover, the biopolymers contained in the residual fibrous mass are beneficial for the soil carbon sequestration of agricultural soils. Nevertheless, surplus fibrous material, especially cellulosic material could be extracted and used for pulp and paper production, while lignin would still be available to supplement farm fertilizer.

2.3 Biopulping using white rot fungi

In the pulp and paper industry, plant biomass, mainly wood, is chemically and mechanically treated to produce paper pulp. This process called pulping has been optimised throughout the centuries but in terms of sustainability of raw materials, chemical and energy usage further

improvement, e.g. diversification of the pulp feedstock, is desirable.[112, 113] Cellulose and hemicelluloses are separated from the other wood components by employing chemicals (NaOH and Na_2S for Kraft pulping)[113] followed by oxygen, hydrogen peroxides, ozone or hypochlorite treatment in order to remove the residual quantities of lignin.[114, 115] The main side product is lignin, which is commonly incinerated to fuel pulp mills[116] but more recently studied as alternative raw material to produce materials.[117, 118]

Biopulping could serve as a pre-treatment for traditional pulping methods aiming to ease the elimination of lignin from lignocellulosic feedstock. During the biopulping process lignin is not removed by chemicals but by enzymatic processes often followed by chemical treatment.[119] Different fungal and bacterial species excrete a wide range of enzymes, which are able to depolymerise lignin. White-rot fungi are prominent lignin-degrading microbial organisms especially in the context of biopulping.[120] The term white-rot refers to the whitish appearance and texture of the remaining wood.[121] This group of fungal species are known to be able to use lignin as carbon source and to convert it into CO_2 and water-soluble components. During fungal growth the enzymes are excreted into the extracellular environment enabling disintegration of lignin.[122] Depending on species and, therefore, on the enzymatic consortium it is possible to achieve selective degradation of lignin or the lignin degradation alongside other wood biopolymers.[123] The main enzymes that are responsible for cleaving the various lignin linkages are lignin peroxidases, laccases and manganese-dependent peroxidases.[124] Around 10,000 species of different white rot fungi exist, which can be chosen to study delignification. Some of the most explored groups are *Pleurotus* fungi (*Pleurotus ostreatus*),[125] *Ganodermataceae* (*Ganoderma lucidum*),[126, 127] *Phanerochaetaceae* (*Phanerochaete chrysosporium*)[128, 129] and *Poliporaceae* (*Trametes versicolor*).[123] For example *Pleurotus ostreatus* is known to excrete only manganese-dependent peroxidases. Using pineapple leaf fibres as substrate *P. ostreatus* was able to decrease the lignin content by 63%, whereas on the same substrate *Trametes versicolor,* excreting lignin peroxidases relying on a free radical mechanism, was able to remove only 29% of lignin.[125]

Biopulping aims at decreasing pulping time, kappa number, which indicates the amount of residual lignin in the pulp and increasing the mechanical properties of produced paper. For example, treatment of agricultural residue, such as oil palm trunk chips treated with *T. versicolor* for 28 days was able to decrease the kappa number by 35%.[130] Yadav *et al.* showed that biopulping of an eucalyptus-poplar wood mix with *Ceriporiopsis subvermispora* could significantly reduce the energy and the chemical load during pulping.[131] By using the same fungal species Akthar *et al.* were able to reduce the energy consumption by 37% and 47% for loblolly pine (*pinus taeda*) and aspen wood chips, respectively. This fungal wood pre-treatment also resulted in an increase in burst and tear index of paper produced from this pulp by 22% and 119%, respectively.[132] A brightness reduction of the resulting paper produced from fungal treated wood was observed, originating from the presence of fungal biopolymers.[133] Although this is considered to be a disadvantage of biopulping, retaining the chitin-β-glucan complex yields a more hydrophobic surface compared to chemical pulping presenting a natural in-situ coating possibility of cellulosic fibres.[134] However, the rather slow process as well as the specific conditions required for the fungal growth, such as temperature and relative humidity, renders fungal pre-treatments challenging on large scale. Nevertheless, *Scott et al.*[135] showed that a minimum of 30% electrical energy can be saved when using wood chips treated 2-week with fungi (*C. subvermispora*). 18% of energy saving was achieved using the same fungal species on *E. grandis* wood chips.[133]

2.4 Structural biopolymers of fungal biomass

During biopulping fungal biomass growths on the lignocellulosic biomass. Similar to the function in wood, chitin and its derivative chitosan are the structural cell wall polymer found in fungi and the exoskeletons of crustaceans and insects.[136] Chitin is a linear polysaccharide composed of (1-4)-linked-2-acetamino-2-deoxy-β-D-glucopyranose units, while chitosan is composed of composed of (1-4)-linked-2-amino-2-deoxy-β-D-glucopyranose units. Both chitin and chitosan are structurally similar to cellulose having an acetamino group in C2 position for chitin or an amine group for chitosan.[137] The most important difference between chitin and

chitosan is their water solubility.[138] Chitin and chitosan are easily processed into gels, membranes, beads and microparticles and found applications in wastewater treatment[139, 140], the food industry and as packaging material[141, 142] and as scaffold for tissue engineering.[143] The source and the extraction process of chitin/chitosan determine their properties.[144] The main sources of commercial chitin are crustaceans, hence the extraction process includes demineralisation with HCl, deproteination with NaOH and decolourisation using oxidative or organic chemicals at elevated temperatures. Alternatively, ionic liquids may allow for a more controlled procedure and final product.[145] In the context of sustainability, recently, fungal biomass has gained considerably importance due to fast growth, cheap growing conditions and mild chemical extraction procedure possible compared to crustaceans because no minerals present need to be removed.[146] The major difference between crustacean chitin and fungal chitin is that in fungi chitin occurs as chitin-glucan complex, in which glucan is chemically grafted to chitin.[147] Different chitin-glucan structures were observed in different fungal species but its general structure constitutes of β-glucan constituted of a (1,3-β-linked) backbone with 1,6-linked branches (**Figure 4**) attached to chitin.[148] The amorphous glucan network is responsible for ductility and flexibility in fungal chitin structures. Papers produced from chitin fibrils extracted from *D. confragosa* (98.9 wt% glucan) exhibited tensile strength of 65 MPa, elastic modulus of 1.2 GPa and elongation to break of 13%, whereas chitin papers produced from nanofibrils extracted from *A. bisporus* (42.6 wt% glucan) were stiffer (6.9 GPa elastic modulus) and much stronger (205 MPa) at the expense of strain-to-failure.[149]

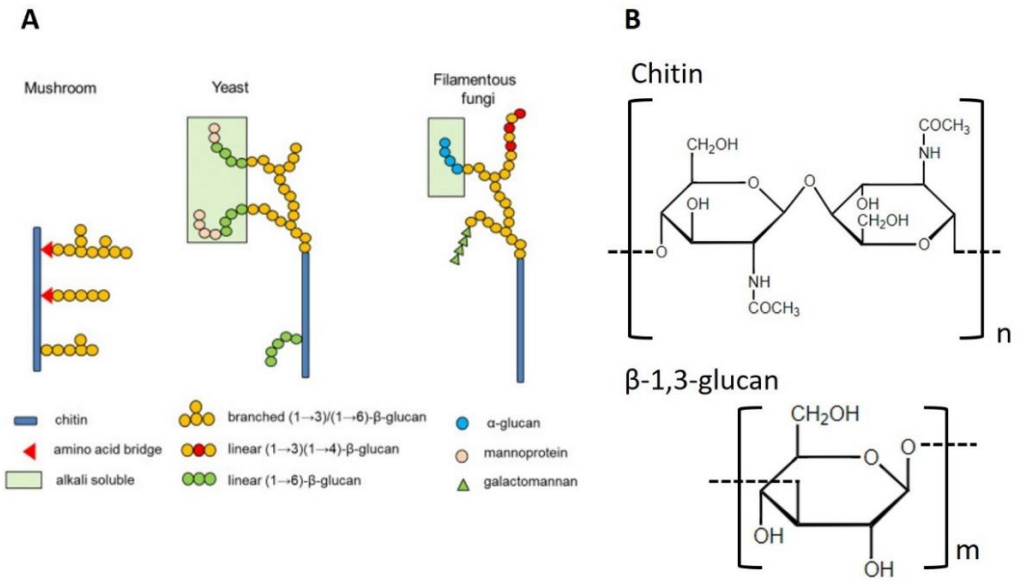

Figure 4 Structure of chitin-glucan complex from different sources (mushroom, yeast, filamentous fungi).[148] (Link in: https://doi.org/10.1021/acs.biomac.9b01141, accessed 25.08.22, open access under the terms and conditions of the Creative Commons Attribution (CC BY) license) (A). Chemical Structure of Chitin and β-1,3-glucan (B).

3 Materials and Methods

3.1 Materials

Elephant manure was kindly provided by Zoo Vienna “Tiergarten Schönbrunn” and fresh horse manure by Reitclub Tulln (Tulln, Austria). *Pleurotus ostreatus* and *Trametes versicolor* fungal cultures were obtained from Tyroler Glückspilze® Mushroom Production Center GmbH (Austria). The inoculum for biogas experiments was a mix of two inocula kindly provided by biogas plants located in Margarethen am Moos and Ziersdorf (Austria). Sodium hydroxide (NaOH, 99.6%), sodium sulphite (Na_2SO_3 99%), microcrystalline cellulose, peracetic acid (38-40%) and sulfuric acid (H_2SO_4, 95%) were purchased from Sigma Aldrich, sodium hypochlorite (NaOCl, 14% active Cl) from W. Neuber’s Enkel (Austria), hexane (≥95%,) from Honeywell Research Chemicals (Austria), decahydronaphthalene from Merck (Darmstadt, Germany). Neutral detergent solution concentrate, acid detergent solution powder, forage bags and triethylene glycol were purchased from Ankom Technology (USA). Ascorbic acid was purchased from Nutribiotic Immunity (USA). Kraft birch pulp was kindly provided by Prof. Eero Kontturi (Aalto University).

Sugar recovery standards for carbohydrate analysis were prepared from L-(+)-arabinose (Merck 101492), D-(+)-galactose (Merck 3455), D-(+)-glucosamine hydrochloride (Sigma-Aldrich G4875), D-(+)-glucose (VWR 10117 HV), D-(+)-mannose (Merck 4440), L-(+)-rhamnose (Merck R3875) and D-(+)-xylose (Merck 108689). Deionized water was used for all experiments.

3.2 Methods

3.2.1 Biogas production and substrate degradation experiments

Volatile solid content (VS), ash content and dry mass (DM) of both types of manure was determined prior to utilisation as feedstock. 3 g of sample (m_{wet}) were dried at 105°C for 24 h

(m_{dry}) and subsequently heated to 550°C for 3h ($m_{550°C}$). DM, VS and ash content were calculated according to equations 1-3:

$$\mathrm{DM\ (wt\%)} = \frac{m_{dry}}{m_{wet}} \cdot 100 \quad (1)$$

$$\mathrm{ash\ (wt\%)} = \frac{m_{550°C}}{m_{dry}} \cdot 100 \quad (2)$$

$$\mathrm{VS\ (wt\%)} = 100 - \mathrm{ash} \quad (3)$$

Anaerobic digestion experiments of elephant and horse manure were performed according to standard VDI 4630 (VDI – The Association of German Engineers 2016). Eudiometer batch fermenters were filled with inoculum (200 g wet mass) and manure in a mass ratio 3:1 based on volatile solid content and stirred continuously at 37.5°C for 40 days. The biogas production and methane content were monitored and analysed with a portable gas analyser (Dräger X-am 7000, Dräger, Germany) on a daily base. Microcrystalline cellulose was used as control substrate.

Investigation of substrate degradation of lignocellulosic biopolymers present in elephant and horse manure was performed simultaneously to the anaerobic digestion experiments.[150] 500 g of fresh manure was put in forage bags, which were placed into containers filled with 20 kg inoculum. Fermentation residue was sampled after 5, 10, 20, 30 and 40 days of AD, respectively, and washed thoroughly with deionised water. The samples were dried at 60°C until constant weight.

The degradation yield was calculated by the ratio of dried and washed fermentation residue after AD and the DM before biogas production (eq. 4)

$$\mathrm{degradation\ yield\ (\%)} = \frac{\mathrm{mass\ after\ biogas\ production\ (g)}}{\mathrm{mass\ before\ biogas\ production\ (g)}} \cdot 100\% \quad (4)$$

3.2.2 Extraction of cellulosic fibres and fertilizer precursor from elephant manure

20 g of dried elephant manure (DM) or fermentation residue were re-suspended in water to separate fibres from sand and stones by flotation and sedimentation. The recovered fibres were purified from animal and microbial biomass by heating the fibre suspension in 1 L of 0.1 M NaOH for 2 h at 80°C. The fibres were removed from the suspension using a 200 mesh (75

µm) sieve. The liquid phase was neutralised by HCl addition and dried (m_{AL}) to determine the solids content of the extract ($yield_{extract}$) (eq. 5):

$$\text{yield}_{\text{extract}}\ (\%) = \frac{\text{m}_{\text{AL}}\ (\text{g})}{\text{DM}\ (\text{g})} \cdot 100\% \ . \quad (5)$$

The recovered fibre residue was washed with water. The alkaline pre-treated lignocellulosic fibres (4 g dry matter) were bleached for 16 h in 660 mL of a 0.4 M NaOCl solution. The bleached fibres were collected from the bleaching solution over the sieve, subsequently dewatered over a cloth and stored at 4°C prior use. To calculate the yield ($yield_{fibres}$) the dry fibre mass (m_F) was determined after oven drying at 105 °C for 24 h (eq. 6).

$$\text{yield}_{\text{fibres}}\ (\%) = \frac{\text{m}_{F}\ (\text{g})}{\text{DM}\ (\text{g})} \cdot 100\% \quad (6)$$

3.2.3 Extraction of lignocellulosic fibres from horse manure

20 g of horse manure or fermentation residue was treated as described above for elephant manure. The only difference was that the recovered fibres were heated in water for 30 min at 85°C to loosen the fibre structure, which was followed by blending in 600 mL of fresh water for 3 min (Tristar BL-4473 VitaPower 2000) prior to alkaline treatment in 1 M NaOH for 3 h at 65°C. The purified fibres were separated using a 635 mesh (20 µm) sieve*. The liquid residue was neutralised using HCl and the solids content determined using equation 5. The fibres were washed thoroughly and stored at 4 °C until further use. The dry mass (m_F) of the received material was determined and the $yield_{fibre}$ was calculated according to equation 6.

3.2.4 Nanocellulose from elephant/horse manure and fermentation residue

To produce NFC from cellulosic fibres extracted from elephant manure and fermentation residue, a suspension of the extracted cellulosic fibres in water (50 g DM at 3.5 wt%) was blended at 1000 rpm for 4 min (JB 3060, Braun) and passed 10 times through a disk mill (Granomat JP 150, Fuchs, Switzerland). To investigate the influence of energy input on the fibre size and tensile properties, samples of extracted cellulosic fibres from elephant manure without anaerobic digestion were taken after 1, 2, 5, 7 and 10 passes, respectively. The energy

* The original used sieve was lost in lab fire.

consumption was monitored using a high voltage meter (Swissnox SX-3M) and an energy logger (Voltcraft 4000). Kraft birch pulp (10 times ground) was used as reference material.

To produce LNFC from lignocellulosic fibres extracted from horse manure or fermentation residue a suspension of extracted fibres (50 g DM at 3.5 wt%) was blended for 4 min (Tristar BL-4473 VitaPower 2000) and passed through a disc mill (Granomat JP 150, Fuchs, Switzerland) 20 times.[†] The resulting suspensions were dewatered to approx. 2 wt% over a textile cloth and stored at 4°C prior to use.

3.2.5 Extraction of NFC from horse manure

To produce reference nanocellulose from horse manure the produced LCNF from horse manure (3.4 g DM) after 0 days AD was suspended in 600 mL of peracetic acid (4 wt% at pH 4.8). The suspension was stirred at 50 °C for 48 h. The suspension was then centrifuged (10 min at 8000 rpm, Hermle Z36 HK, Germany) and the supernatant discarged. The procedure was repeated and the reaction quenched using ascorbic acid. The suspension was centrifuged and resuspended in water until pH 7. The NFC yield was calculated using equation 6.

3.2.6 Fungal cultivation on elephant manure

Prior to use, freshly collected elephant manure was sterilised in an autoclave (120°C, 90 min) and stored at −11°C. The material was defrosted at room temperature and re-sterilised (120°C, 90 min). Cultures of *P. ostreatus* and *T. versicolor* were pre-inoculated on agar plates (1.2 g agar, 1 g malt extract in 100 g water) for 5 days in the dark at 25°C. 8 mm diameter inoculum disks were stamped from the cultures and placed onto 30 ± 0.03 g (3-4 g dry mass ($m_{pre\text{-}growth}$) sterilised elephant manure. Petri dishes were stored in darkness at 25°C for 4, 8, 12 and 16 weeks. At least six replicates for each cultivation time were plated. The dry mass of each sample pre- and post-growth ($m_{post\text{-}growth}$) was determined across triplicate samples using a micro-balance (Sartorius Cubis®) following oven drying for 24 h at 105°C.

[†] Unfortunately, the disc mill was damaged in a lab incident and had to be repaired, which required a reinstallation a few months later necessitating different settings.

3.2.7 Fungal biomass modified lignocellulosic fibres from elephant manure

After the designated growth time the content of 6 petri dishes of colonised elephant manure were weighed, combined and blended in 500 mL water for 3 min. The suspension was heated to 85°C for 30 min. After cooling to 25°C the suspension was centrifuged for 10 min at 8500 rpm (Hermle Z36 HK, Germany), the supernatant discarded and the recovered fibres reheated in 500 mL of 1 M NaOH at 65°C for 3 h. Repeated centrifugation and redispersion in water enabled neutralisation until pH 7. The extraction yield ($yield_{extraction}$) was calculated using the dry mass $m_{post\text{-}growth}$ and the dry mass of the extracted composite material $m_{post\text{-}extraction}$ (eq. 7):

$$\mathrm{yield_{extraction}}(\%) = \left(\frac{\mathrm{m_{post-extraction}(g)}}{\mathrm{m_{post-growth}\ (g)}}\right) \cdot 100 \quad (7)$$

The total yield ($yield_{composite}$) was calculated based on the dry mass (eq. 8):

$$\mathrm{yield_{composite}}(\%) = \left(\frac{\mathrm{m_{post-extraction}(g)}}{\mathrm{m_{pre-growth}\ (g)}}\right) \cdot 100 \quad (8)$$

3.2.8 Chemical analysis of lignocellulosic material

Lignocellulosic biomass (elephant or horse manure, fermentation residue or extracted (ligno)cellulosic fibres) were characterised with regards to their ash content (ash_f) and structural biopolymer content: cellulose, hemicellulose and acid detergent lignin (ADL). The dried raw manure or fermentation residue was first ground using a cryomill (CryoMill, Retsch, Germany) (Elephant manure: pre-cooling at 5 Hz (2 min), grinding at 28 Hz (14 min); horse manure: pre-cooling at 5 Hz (1 min), grinding at 20 Hz (8 min)). The analysis of the biopolymer content was performed according to the method of Naumann and Bassler.[151, 152] The water content (w) of every sample was determined by Karl Fischer titration (Mettler Toledo V20, Columbus, Ohio, USA) and the mass of the dry sample (m_1) calculated based on sample weight (m_s) (eq. 9).

$$\mathrm{m_1(g)} = \mathrm{m_s} - (\mathrm{m_s} \cdot \mathrm{w}) \quad (9)$$

Neutral detergent fibre (NDF): (hemi)cellulose and lignin content

0.5 g sample (m_s) was immersed in 50 mL NDF solution (30 g neutral detergent powder and 5 mL triethylene glycol in 500 mL water). 1 mL decahydronaphthalene and 0.25 g sodium sulphite were added to each sample and the suspension was boiled for 110 min under reflux. The residual was filtered using a filter crucible (Por. 3) and washed with water and acetone. The filter cake was dried for 24 h at 105 °C before determining the mass (m_2). The NDF content was calculated (eq. 10):

$$\mathrm{NDF\ (wt\%)} = \frac{m_2\ (g)}{m_1 (g)} \cdot 100\% \quad (10)$$

Acid detergent fibre (ADF): cellulose and lignin content

0.5 g of sample (m_s) was treated with 50 mL ADF solution (10 g acid detergent powder in 500 mL 0.5 M sulphuric acid) and 0.5 mL decahydronaphthalene for 110 min under reflux. The material was filtered using a filter crucible (Por. 3) and washed thoroughly with water and acetone. The filter cake was dried over night at 105°C and its dry mass determined (m_3). The ADF content was calculated (eq.11):

$$\mathrm{ADF\ (wt\%)} = \frac{m_3\ (g)}{m_1\ (g)} \cdot 100\% \quad (11)$$

Acid detergent lignin (ADL): lignin and ash content

The filter cake from ADF analysis was resuspended in 8 mL of 72% H_2SO_4 and stirred shortly with a glass stirrer. Additional 7 mL of 72 % $H2SO_4$ were added. The mixture was stirred every 30 min manually. After 3 h, the suspension was filtered using a water-jet pump and washed thoroughly with water. The residue was dried for 24 h at 105°C and the mass of the filtrate (m_4) determined. The ADL content was determined as follows:

$$\mathrm{ADL\ (wt\%)} = \frac{m_4\ (g)}{m_1 (g)} \cdot\ 100\% \quad (12)$$

To determine the ash content the filtrate was heated in a muffle furnace for 5 h: The oven was set to reach 550°C within 3 h and held 550°C for another 2 h. The crucible filters were weight again after cooling down to (m_5). The ash content was determined as follows:

$$\text{ash}_f\ (\text{wt\%}) = \frac{m_5(\text{g})}{m_1(\text{g})} \cdot\ 100\% \quad (13)$$

Cellulose, hemicellulose and lignin contents were calculated based on equation 14, 15 and 16

$$\text{Cellulose}\ (\text{wt\%}) = \frac{(\text{ADF}-\text{ADL})}{(100-\text{ash}_f)} \cdot 100 \quad (14)$$

$$\text{Hemicellulose}\ (\text{wt\%}) = \frac{(\text{NDF}-\text{ADF})}{(100-\text{ash}_f)} \cdot 100 \quad (15)$$

$$\text{Lignin}\ (\text{wt\%}) = \frac{\text{ADL}}{(100-\text{ash}_f)} \cdot 100 \quad (16)$$

Analysis of lignin content of fungal biomass modified lignocellulosic fibres

Acid-insoluble lignin (AIL) was determined according to standard T222 om-02 by acid hydrolysis. The extracted material was dried at 105°C and ground to a particle size <1 mm (Universal mill, UM620, Karcher, Germany). To 0.3 g sample (m_s) 3 mL of 72% H_2SO_4 was added and stirred for 1 h at 30°C. Subsequently, the mixture was diluted to 4% by adding 84 mL water and heated to 121°C for 60 min. The mixture was filtered over a filter crucible (Por. 3) and washed with water until pH 7. The filter cake was dried at 105°C and weighed (m_{AIL}). Finally, the acid-insoluble ash content was determined by heating the sample at 500°C for 3 h ($m_{ashfibre}$). According to standard T211 om-02, correction for ash contained in lignin was performed using equation 17.

$$\text{AIL}\ (\text{wt\%}) = \frac{m_{\text{AIL}} - m_{\text{ashfibre}}\ (\text{g})}{m_1\ (\text{g})} \cdot 100\% \quad (17)$$

Determination of extractives content of fungal biomass modified lignocellulosic fibres

0.5 g of fungal biomass modified lignocellulosic fibres extracted from elephant manure cultivated with fungal species were dried at 105°C for 12 h and weighed (m_{dry}). Dry samples were then extracted using Soxhlet extraction with 200 mL of hexane for 6 h (4-5 cycles/h). Subsequently, the samples were dried again at 105°C for 12 h and weighed (m_{ex}). The extractives content was calculated using equation 18.

$$\text{Extractives content}\ (\text{wt\%}) = \left(1 - \frac{m_{\text{dry}}\ (\text{g})}{m_{\text{ex}}(\text{g})}\right) \cdot 100\% \quad (18)$$

3.2.9 Nano/Paper production

Nano/Papers with a grammage (G) of 100 g m^{-2} were produced by blending 1.22 g (DM) extracted (nano)ligno/cellulosic fibres for 3 min at 1000 rpm in 300 mL of water. The suspension was filtered over a Büchner filter funnel (VitraPOR, 100 mL, Por. 3, Robu, Germany) onto a filter paper (VWR 413, 125 mm in diameter). The filter cake was removed from the filter paper and pressed twice for 10 s between two blotting paper sheets at 20°C at 20 kN force using a hydraulic press (Model 412 6CE, Carver, USA) and subsequently between two fresh sheets of blotting paper and one sheet of baking paper at 120°C for 15 min at 20 kN force.

3.2.10 Physical and mechanical properties of the produced paper materials

Physical properties

Dog bone specimens (1BA, EN ISO 527-2) of (NFC) papers produced from fibres extracted from elephant manure and fermentation residue, as well as fungal biomass modified lignocellulosic fibres with 75 mm length and 5 mm parallel width were punched using a cutting press (Zwick ZCP 020 Manual Cutting Press, Zwick, Germany). For LCNF papers produced from extracted horse manure and fermentation residue specimens of 2 mm width and 25 mm overall length were cut (1BB, EN ISO 527-2). A digital micrometer (AnyiMeasuring) was used to measure the thickness (d) of the test specimens. The envelope density ρ_e was calculated by the ratio of G and d (eq. 19).

$$\rho_e\ (\mathrm{g\,cm^{-3}}) = \frac{G}{d} \tag{19}$$

The skeletal density ρ_s of the papers was determined by helium gas displacement pycnometry (Micromeritics AccuPyc II 1340) with a chamber volume of 1 cm^3. The porosity (Φ) was then calculated from the envelope and skeletal densities (eq. 20).

$$\Phi\ (\%) = \left(1 - \frac{\rho_e}{\rho_s}\right) \cdot 100 \tag{20}$$

Mechanical properties

The specimens were conditioned in the laboratory at 23 ± 5 °C and RH 50 ± 3% for 24 h. The tensile properties were determined at a testing speed of 1 mm min^{-1} using a universal test frame (Model 5969, Dual Column Universal Testing System, Instron, Germany) equipped with a 1 kN load cell and a non-contact video extensometer (Gig ProE, iMETRIUM, Bristol, UK). The samples were clamped between blotting paper to avoid perforation by the metal clamps. The tensile strength (σ) was determined by dividing the maximum load by the cross-sectional area of the specimen. The strain to failure (ε) was defined by the strain corresponding to the maximum load. The elastic modulus (E) was determined as secant between strength values separated by 0.2% strain in the linear elastic region of the stress-strain curve.

Morphology

Images of the NFC papers from elephant manure were obtained by scanning electron microscopy (SEM) (Zeiss Supra 55VP, accelerating voltage of 2 kV, working distance of 7.2 mm). Thin sheets of LCNFs extracted from horse manure and fermentation residue were produced by dilution of the suspension to 2 wt% and drying in a petri dish at 105 °C over night. 10 x 10 mm samples were coated with a 10 nm gold layer (Leica scd 050/EM QSG 100, 60 mA for 67 s). The dimensions of at least 40 fibres were averaged for determining the fibre diameter (ImageJ 1.53e, https://imagej.nih.gov/ij)

Surface properties

The wettability of the produced paper materials was determined by measuring static water contact angles using the sessile drop method. Water droplets (2 to 5 µL) were placed on the paper surface and monitored using a drop shape analyser (DSA30, Krüss GmbH, Germany). At least 50 single droplets were placed on the samples and measurements taken after 5, 30, 60 and 120 s after dosing.

Water retention

The water retention of the produced LCNF papers produced from extracted horse manure and fermentation residue was determined by cutting 20 mm x 5 mm samples, which were conditioned at 20 °C and 44% RH for 24 h. The mass of the samples were determined before (m_6) and after water immersion for 24 h (m_7). It was taken care to remove excess water with a paper cloth. The water adsorption capacity was calculated using equation 21.

$$w_{H2O}(wt\%) = \frac{m_7 - m_6}{m_6} \cdot 100 \quad (21)$$

4 Results and Discussion

4.1 Biogas production from manure

Elephant and horse manure were used as substrate for biogas production. To estimate the biogas production capacity of the substrate volatile solid (VS) and ash contents of both manure species were determined. Horse manure had a VS content of 73 wt%, which was significantly lower than the VS of elephant manure (89 wt%). Accordingly, the ash content (27 wt%) was significantly higher than of elephant manure (5 wt%). The higher volatile solid content of elephant manure portrays the higher share of organic biomass, which can be converted into biogas and methane. One explanation for the high ash content of horse manure could be sand and stones, which were unavoidable during sample collection. Using elephant manure as a substrate 399 $L_N\ kg^{-1}_{vs}$ of biogas with a methane concentration of 61% (244 $L_N\ kg^{-1}_{vs}$) were produced (**Figure 5**). Using horse manure only 207 $L_N\ kg^{-1}_{vs}$ biogas and 135 $L_N\ kg^{-1}_{vs}$ methane (65 %) were obtained at the same fermentation time. During the first 10 days 262 $L_N\ kg^{-1}_{vs}$ of biogas from elephant manure was produced, which equals 66% of the total biogas production. Using horse manure after 10 days 49% of total biogas was produced and after 16 days already 89%.

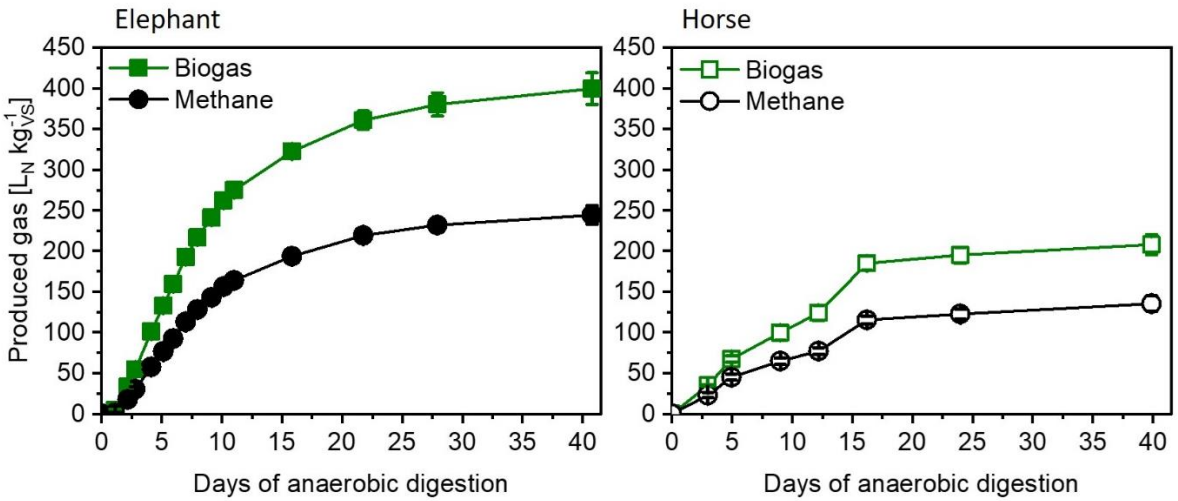

Figure 5 Biogas (square, green) and methane (circle, black) production from elephant manure (filled) and horse manure (hollow) substrate over a course of 40 days.

During anaerobic digestion the organic fibrous material containing cellulose, hemicellulose and lignin is converted into biogas, resulting in a loss of substrate material. The degradation yield of elephant manure (85 wt%) and horse manure (83 wt%) prior to AD is attributed to the loss of material caused by washing (**Table 2**), which was necessary to produce the reference material. Elephant manure degraded faster during the first 10 days of AD (from 85 wt% to 46 wt%) compared to horse manure of which 68 wt% of washed and dried fibrous material was obtained after 10 days. After 40 days of AD only 34 wt% fibrous material could be obtained from elephant manure, while almost double the amount remained when using horse manure substrate. The fibre composition changed significantly over the course of 40 days of AD. The washed and dried fibres of elephant manure had cellulose, hemicellulose and ADL contents of 38 wt%, 34 wt% and 18 wt%, respectively. In this context it is worth mentioning that elephant manure samples collected at different times (April, June or January) resulted in significantly different fibre composition due to the changes in feed for the elephants. The material collected in January had the highest ADL content (37 wt%). The raw material collected in June exhibited an ADL content of 22 wt% and the highest cellulose content of 68 wt%.

The content of hemicellulose and cellulose in elephant manure decreased by 15% and 9%, respectively, over the course of 40 days, while the lignin content increased by 68%. Using horse manure as feedstock a similar trend for cellulose and hemicellulose degradation was observed (hemicellulose from 21 wt% to 13 wt% and cellulose 48 wt% to 44 wt% over the

course of 40 days). The lignin content increased by 50%. Cellulose and hemicellulose are preferably converted by the microbes during AD due to their higher accessibility resulting in a up-concentration of lignin in the fermentation residue. The results showed that anaerobic digestion can be used as a treatment to generate an additional value from manure, i.e., biogas and methane, but also is a means to adjust the lignin, hemicellulose and cellulose ratio in the fermentation residue produced.

Table 2 Degradation yield, fibre ash content (ash_f), fibre composition of cellulose (Cel), hemicellulose (HCel) and ADL of washed horse and elephant manure and fermentation residue after 5 to 40 days of anaerobic digestion.

	Elephant manure					Horse manure				
AD	Degradation yield	ash_f	HCel	Cel	ADL	Degradation yield	ash_f	HCel	Cel	ADL
[d]	[wt%]	[wt%]	[wt%]			[wt%]	[wt%]	[wt%]		
0	85*	4.5	34.2	38.0	18.8	83*	7.0	20.5	48.3	27.9
5	66	6.1	38.7	38.3	21.1	74	5.7	19.6	48.6	28.4
10	46	8.5	29.5	35.6	30.4	68	6.7	17.4	48.0	30.1
20	36	8.8	27.8	34.9	30.3	63	5.5	16.4	45.7	32.3
30	36	8.8	24.5	34.2	28.8	59	11.5	15.2	43.7	40.6
40	34	7.9	29.0	34.4	31.7	60	13.4	12.9	44.2	41.9

*fibre yield of washed manure

4.2 Extraction of nano(lingo)cellulosic fibres from manure and fermentation residue

4.2.1 Cellulosic fibres from elephant manure and fermentation residue

Elephant manure and fermentation residue were used as raw material for the extraction of cellulosic fibres. The extraction procedure was performed in two steps. First an alkaline treatment (0.1 M NaOH) for the removal of proteins and keratinized tissue followed by bleaching with NaOCl (0.4 M) for the removal of lignin and to receive white fibres. The total yield of cellulosic fibres was significantly influenced by the anaerobic digestion process. 41 wt% of cellulosic fibres could be extracted from as received elephant manure, which was already halved after 5 days of AD. After 20 days of AD only 12 wt% of fibrous material were obtained. This yield did not change anymore when extending fermentation time for a further 20 days.

A fertilizer precursor was obtained of the dried and neutralised liquid after the alkaline treatment of manure and fermentation residue, respectively. Yields of the obtained precursor ranged from 25 wt% for material without anaerobic digestion to 11 wt% after 40 days of AD.

To serve as fertilizer carbon and nitrogen contents are crucial. The dry matter content of the liquid fermentation residue was composed of 20 to 28 wt% carbon and contained <1 wt.% nitrogen. In accordance with the fertilizer regulation (BGBl II Nr 100/2004) this fullfills the definition of a fertilizer. By purification the mineral content of the fertilizer precursor can be optimised to meet the soil's needs. This will not be discussed further as it is outside the scope of this work.

The cellulosic fibres extracted from elephant manure and fermentation residue were used for the production of paper and their tensile properties were assessed (**Table 3**). While the papers produced from fibres from elephant manure had a tensile strength of 60 MPa, σ increased first to 74 MPa after 5 and 10 days of AD but decreased to approx. 30 MPa for papers produced from fibres extracted after 20, 30 and 40 days of AD. Elastic moduli and strains to failure followed this trend and decreased by 37% (9.1 GPa for papers produced from fibres after 0 days AD to 5.7 GPa for papers produced from fibres after 40 days) and 52% (from 1.22% to 0.58%), respectively. Lignin accumulated during the AD process rendering the bleach concentration insufficient to remove all lignin from the fermentation residues resulting in a significant increase of the lignin content (from 2.3 wt% to 10.4 wt%) on the cellulosic fibres over the course of 40 days. The higher fraction of aromatic structures in lignin remaining on the fibres disturbed the formation of hydrogen bonds between the cellulose fibrils formed during hot pressing resulting in lower connectivity of the fibrils, which in turn resulted in lower mechanical properties.

Table 3 Fibre and extract yields, fibre ADL content (ADL) and tensile properties (tensile strength (σ), elastic modulus (E) and strain to failure (ε)) of fibres extracted from elephant manure and fermentation residue after 5 to 40 days of anaerobic digestion.

AD	$Yield_{fibre}$	$Yield_{extract}$	ADL	σ	E	ε
[d]	[%]	[%]	[wt%]	[MPa]	[GPa]	[%]
0	41	25	2.3	60.4 ± 4.9	9.1 ± 0.7	1.22 ± 0.12
5	21	24	4.3	74.0 ± 2.3	11.1 ± 1.2	1.37 ± 0.11
10	17	16	5.2	66.2 ± 6.8	9.7 ± 0.6	0.92 ± 0.08
20	12	13	8.3	32.2 ± 2.3	6.2 ± 0.8	0.68 ± 0.04
30	12	12	8.9	31.2 ± 8.4	5.7 ± 0.8	0.70 ± 0.13
40	12	11	10.4	27.4 ± 3.4	5.7 ± 0.3	0.58 ± 0.07

4.2.2 Nanofibrillation of cellulosic fibres from extracted elephant manure and fermentation residue

Defibrillation of the extracted fibres from elephant manure and fermentation residue was performed to improve their mechanical properties and to produce NFC fibres. Cellulosic fibres extracted from elephant manure without anaerobic digestion were used to investigate the influence of the energy input, i.e. number of grinding cycles, on the fibre diameter and the mechanical properties of produced papers (**Figure 6**). Already after 1 grinding cycle the tensile strength doubled (from 64 MPa to 126 MPa), while the elastic modulus increased from 7.1 GPa to 12.3 GPa. With increasing number of grinding cycles the tensile strength continuously increased up to 196 MPa for material ground 10 times, while the paper tensile modulus did not increase further with increasing number of grinding cycles. The strain to failure significantly increased to 3.6%. Despite bigger fibres with diameter of 7 to 0.5 µm still being present in the paper after 1 grinding cycle, already fibrils in nm range were observed. SEM micrographs of the produced papers prepared using cellulosic fibres after 1, 5 and 10 grinding cycles exhibited an increased homogeneous appearance with increasing number of cycles (**Figure 6**). An increased energy input decreased the fibre width and resulted in a higher homogeneity of the paper and thus in better connectivity between the fibres which all contributed to increased tensile strength. The influence of the variability in composition of the raw material was also obvious in this set of experiments. After the same number of grinding cycles, nanopapers produced from fibrils extracted from elephant manure collected in June and January had a 34% and 51% lower tensile strength then the material collected in April (130 MPa and 96 MPa, respectively). Additionally, the fibre diameter was approx. 50% larger (40 and 44 nm, respectively) than that of fibrils produced from dung collected in April, which had a fibre diameter of 22 nm. AD, irrespectively of the fermentation time, had no influence on the fibre diameter, which remained constant between 22 and 32 nm after defibrillation using 10 grinding passes.

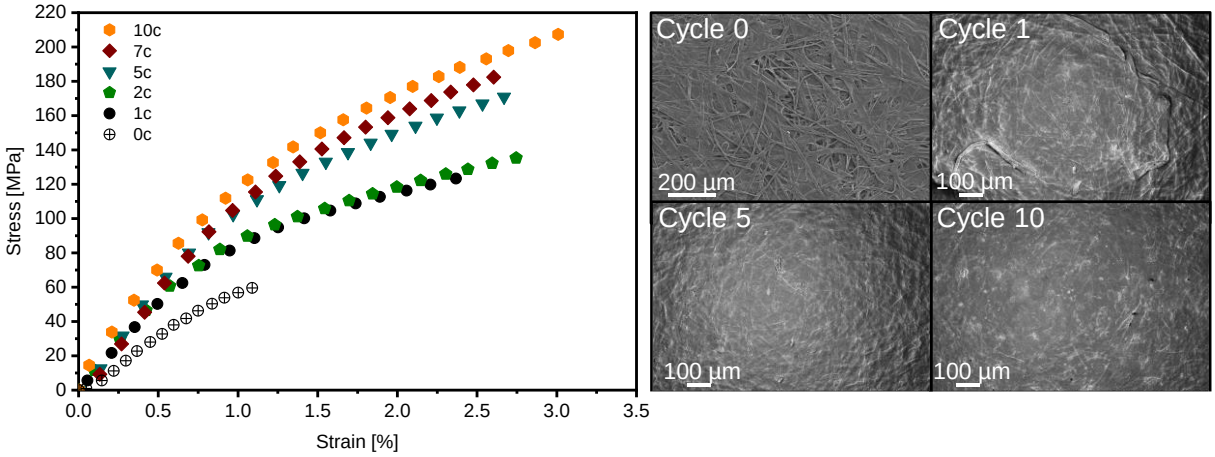

Figure 6 Representative stress strain curves of nanopapers produced from cellulosic fibres extracted from elephant manure and different grinding cycles (Link in: accessed 24.10.22, open access under the terms and conditions of the Creative Commons Attribution (CC BY) license https://doi.org/10.1021/acssuschemeng.1c05175). Corresponding SEM micrographs of papers made using fibrils obtained after 0, 1, 5 and 10 grinding cycles are shown on the right side.

The tensile strength of papers produced from defibrillated fibres (10 times passes through the grinder) extracted from elephant manure of 0 to 40 days AD decreased by 47% (196 MPa vs 125 MPa, respectively). The elastic moduli were unaffected by the anaerobic digestion process, while the strain to failure decreased by 56% (3.6% vs 1.6%). The reduction in tensile strength and strain to failure was also associated with the up-concentration of lignin in the material but was partially mitigated by the fibrillation process during grinding. The molecular weight of the extracted lignocellulosic fibres did not follow any significant trend and the influence of the biological variability of the raw material is therefore more pronounced than the influence of AD time. The energy consumption for NFC production increased with increasing number of grinding cycles. For the first cycle 0.3 kWh kg^{-1} was needed and after 10 cycles the total energy consumption was 5.2 kWh kg^{-1}. This value is at the lower end of energy requirements for NFC production reported in the literature, which ranged from 5 to 30 kWh kg^{-1}.[47] This shows the great potential of manure for the production of both, biogas and NFC from fermentation residue. While most of biogas and methane was produced after 10 days, nanopapers made from defibrillated fibres extracted from fermentation residue at this AD time had a tensile strength of 185 MPa, an elastic modulus of 14 GPa and a strain to failure of 2.2%, which is comparable to nanopapers produced from wood pulp. However increasing the biogas production time to 20 days enables to obtain 34% and 36% more biogas and methane,

respectively, mitigating the energy consumption used while defibrillation. By adaptation of the chemical extraction process lignin could be removed in a more efficient way leading to higher purity of the received NFC.

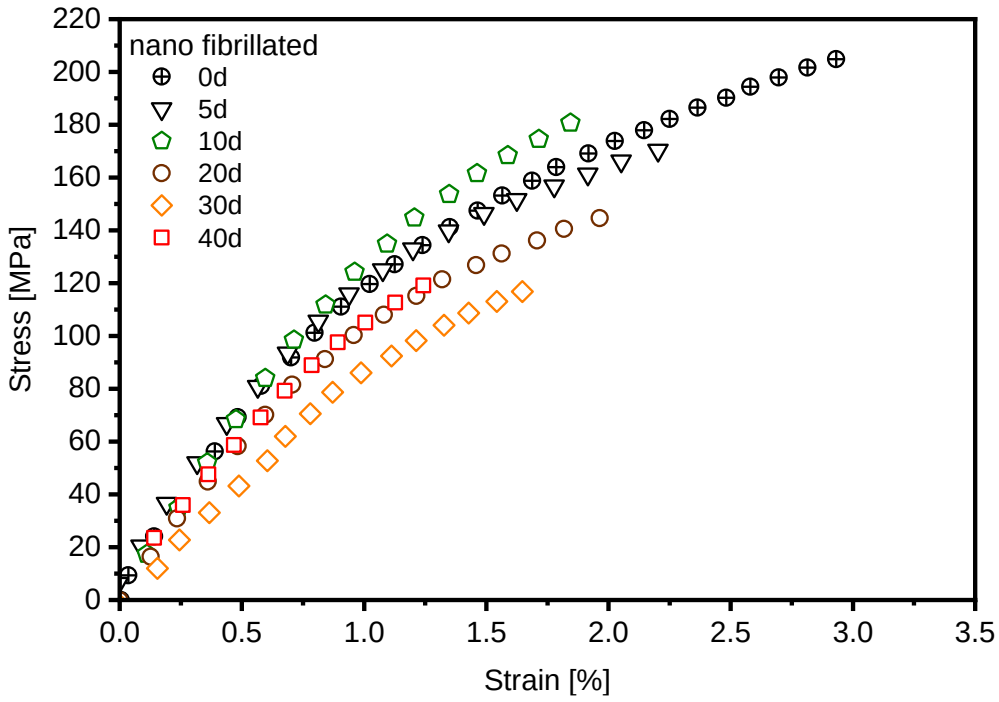

Figure 7 Representative stress strain curves of nanopapers produced from cellulosic fibres extracted from elephant manure and fermentation residue of 5 to 40 days of AD (Link in: accessed 24.10.22, open access under the terms and conditions of the Creative Commons Attribution (CC BY) license https://doi.org/10.1021/acssuschemeng.1c05175).

Table 4 Tensile properties (tensile strength (σ), elastic modulus (E) and strain to failure (ε)) of NFC papers produced from cellulosic fibres extracted from elephant manure and fermentation residue after 5 to 40 days of AD.

AD [d]	Cycles	σ	E	ε	Fibre diameter
		[MPa]	[GPa]	[%]	[nm]
0	1	123 ± 6	12.3 ± 0.8	2.1 ± 0.3	36 ± 17
0	2	142 ± 11	12.6 ± 0.9	3.2 ± 0.4	32 ± 24
0	5	171 ± 23	11.7 ± 0.7	2.9 ± 0.5	27 ± 6
0	7	181 ± 16	11.9 ± 1.0	3.2 ± 0.6	33 ± 14
0	10	196 ± 12	11.6 ± 1.2	3.6 ± 0.6	22 ± 4
5	10	173 ± 7	14.5 ± 0.6	2.7 ± 0.6	22 ± 7
10	10	185 ± 7	14.2 ± 1.0	2.2 ± 0.4	32 ± 8
20	10	144 ± 7	13.5 ± 0.3	1.9 ± 0.2	27 ± 6
30	10	115 ± 5	13.3 ± 1.3	1.7 ± 0.2	27 ± 6
40	10	125 ± 9	12.9 ± 0.6	1.6 ± 0.3	31 ± 6

4.2.3 Lignocellulosic fibres from horse manure and fermentation residue

In order to benefit from the up-concentration of lignin during the AD process, lignocellulosic fibres with a high but tuneable lignin content were produced from horse manure and fermentation residue. Horse manure was anaerobically digested over a course of 40 days followed by washing, alkaline extraction and mechanical defibrillation to obtain LCNF. As anticipated the fibre yield decreased with increasing days of AD. Horse manure without AD had a fibre yield of 41 wt%, which reduced by 32% after 40 days (28 wt%) (**Table 5**).

A fertilizer precursor was also obtained by neutralising and drying the liquid residue after alkaline treatment. The carbon and nitrogen contents were significantly lower than in the fertilizer precursor obtained from elephant manure due to the higher concentration of NaOH had to be used for the extraction and thus the higher amounts of NaCl formed during the neutralisation. The dry matter of the liquid fermentation residue had carbon contents of 1 to 3 wt% and nitrogen contents of approx. 0.1 wt%. By purification, the mineral content of the fertilizer precursor can be optimised to meet the soils needs. This will not be discussed further as it is outside the scope of my work.

During AD and alkaline extraction hemicellulose was removed yielding approx. 10 wt% in all samples. The decrease in cellulose content from 64 wt% to 56 wt% in the fibrous material obtained is caused by cellulose conversion to biogas during AD and not by the extraction process because of the resistance of cellulose to alkaline treatment. Simultaneously, the lignin content increased by 26% from 22.7 wt% to 28.7 wt% already after 20 days of AD. During anaerobic digestion lignin is not converted into biogas and is accumulated in the fermentation residue. During the first 20 days of the anaerobic digestion most of the biogas is produced, while continuing the AD process does not increase the amount of lignin in the material any more (**Table 5**). Proceeding AD altered the lignin to cellulose ratio giving the additional possibility to produce material with a variable ratio of those components. The increased lignin content in the LCNFs caused higher hydrophobicity of the produced LCNFs resulting in a lower water uptake (W_{H_2O}). LCNF papers produced from fibres extracted from as-received dung had a water uptake of 125 wt%, which decreased after 40 days of AD by 30% (87 wt%) (Table 5).

A nanopapers produced from bleached horse manure (0b) had a 34% higher water uptake compared with the non-bleached equivalent and even 92% then the LCNF paper produced from extracted fermentation residue after 40 days. The water contact angle on nanopapers produced from fibrils extracted from horse manure and fermentation residue measured directly after droplet deposition ranged between 63° and 75° and showed no correlation with the residual lignin content (ADL), which indicates that lignin is presented on the LCNF paper surface.

Table 5 Fibre yield, fibre ash content (ash_f), fibre composition (cellulose (Cel), hemicellulose (HCel) and lignin (ADL)), water retention (W_{H2O}) as well as mechanical properties (tensile strength, elastic modulus and strain to failure) of LCNF nanopapers produced from horse manure and anaerobic digestion residue.

AD	$Yield_{fibre}$	ash_f	Cel	HCel	ADL	W_{H2O}	σ	E	ε
[d]	[%]	[wt%]		[wt%]		[wt%]	[MPa]	[GPa]	[%]
0	41	1.6	64.5	11.7	23.1	125 ± 21	65 ± 16	8.0 ± 0.7	1.2 ± 0.3
5	39	1.1	64.2	12.3	22.8	112 ± 11	91 ± 9	7.7 ± 1.0	1.6 ± 0.3
10	34	1.0	61.6	10.0	26.4	110 ± 6	88 ± 6	7.1 ± 1.0	1.9 ± 0.2
20	32	0.5	58.9	10.4	29.1	93 ± 6	58 ± 11	7.9 ± 0.7	1.0 ± 0.1
30	27	0.9	57.5	10.3	29.2	84 ± 3	61 ± 11	7.0 ± 1.3	1.2 ± 0.3
40	28	2.1	56.4	11.0	29.1	87 ± 8	45 ± 11	7.0 ± 0.6	0.8 ± 0.3
0b	21	0.2	92.1	6.8	0.7	167 ± 5	130 ± 9	12.6 ± 0.7	1.7 ± 0.7

The variations in lignin content influenced the mechanical properties of the produced nanopapers (**Figure 8**). The tensile strength increased from 65 MPa to 91 and 88 MPa after 5 and 10 days of AD, respectively. LCNF paper produced from fermentation residue after 20 and 30 days of AD still exhibited a tensile strength of approx. 60 MPa. This drop in tensile properties is again attributed to the presence of lignin around the cellulose nanofibrils affecting network formation. The modulus of nanopapers produced from fermentation residue remained constant within the error irrespective of AD time. The strain to failure increased after 10 days of AD (1.9%) and then decreased again with increasing fermentation time 0.8% (40 days). The produced LCNF paper followed the same trend in mechanical properties as NFC paper produced from cellulose extracted from elephant manure and fermentation residue.[19] Nanopapers produced from bleached fibres extracted from horse manure after 0 days AD had a tensile strength of 130 MPa, a modulus of 12.6 GPa and a strain to failure of 1.7%. The removal of lignin allowed for the formation of a higher number of hydrogen bonds between the

cellulose fibrils resulting in a stronger, but stiffer nanopaper compared to nanopapers produced from unbleached fibres extracted from horse manure after 0 AD.

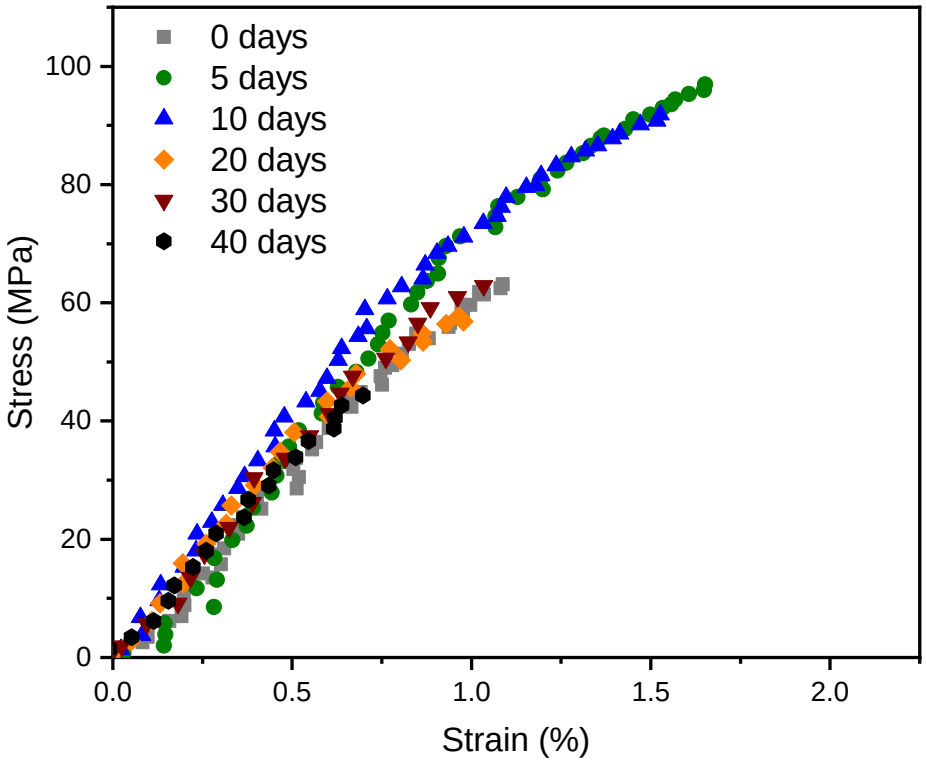

Figure 8 Representative stress strain curves of nanopapers produced from LCNF extracted from horse manure (grey square) and fermentation residue of 5 (green circle), 10 (blue triangle up), 20 (orange diamonds), 30 (red triangle down) and 40 days (black pentagon) of AD.

4.3 Lifecycle assessment of the proposed manure utilisation system

Lifecycle assessment (LCA) was performed to evaluate the environmental impact of the proposed manure utilisation system. Elephant manure was used as model system to study the environmental impact categories when used as raw material for the production of biogas and subsequently NFC from the fermentation residue. Our process was compared to already established biogas and kraft pulp production systems, which were considered in combination (**Figure 9**).[153] Two scenarios were modelled in which two different raw materials were investigated for the production of biogas and NFC. The NFC production based on wood pulp was compared to our approach. To create equality in the base line wood chips scenario a typical Austrian biogas facility using maize silage/pig slurry mixed substrate was added to the pulping process. Hard wood chips were used as source for the NFC production.

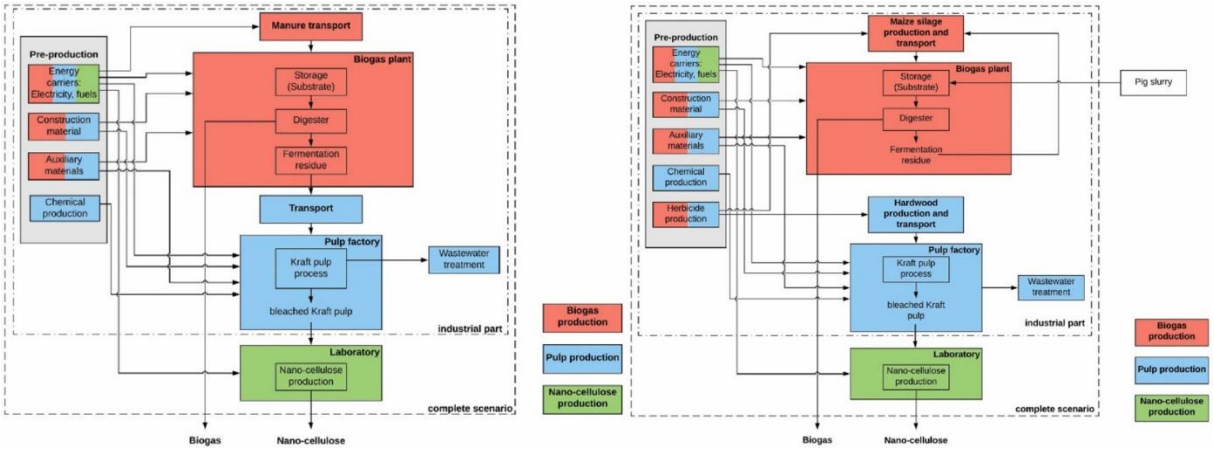

Figure 9 Modelled systems for LCA analysis.[153] Left: manure scenario; right wood chip scenario. (Link in: https://www.sciencedirect.com/science/article/pii/S0301479722006661, accessed 29.09.22, open access under the terms and conditions of the Creative Commons Attribution (CC BY) license)

The relative environmental impact was studied using the following impact categories: Global warming potential (GWP100), freshwater eutrophication potential (FEP), fossil resource scarcity (FRS), terrestrial acidification potential (TAP), human carcinogenic/non-carcinogenic toxicity potential (HCTP, HNCTP) and terrestrial ecotoxicity potential (TEP). For every impact category the highest absolute value of either scenario was set to 100%, the lower number is shown as percentage share (**Figure 10**). LCA results clearly indicated superiority of the manure scenario compared to the wood chips scenario. The environmental impacts of the manure scenario were only ~50% of the wood chips scenario in most of the impact categories, such as global warming potential (GWP100), fossil resource scarcity (FRS) or human non-carcinogenic toxicity potential (HNCTP). The TAP category had the lowest relative impact (17%). The highest contribution was in TEP with 55%. While the biogas production in the manure scenario contributed less than 10% in all impact categories, the NFC production was the main contributor, with the exception of TAP and TEP, to all impact categories – 99.9% of which were caused by the high electricity demand for fibre refining. The biogas production part in the manure scenario contributed less than 10% in the examined impact categories except for GWP and TEP. The highest share of biogas production contribution can be found in TAP in the wood chips scenario (75%), which is due to the fertilization with liquid fermentation

residue using a vacuum tanker and the associated ammonia emissions during production of maize silage.

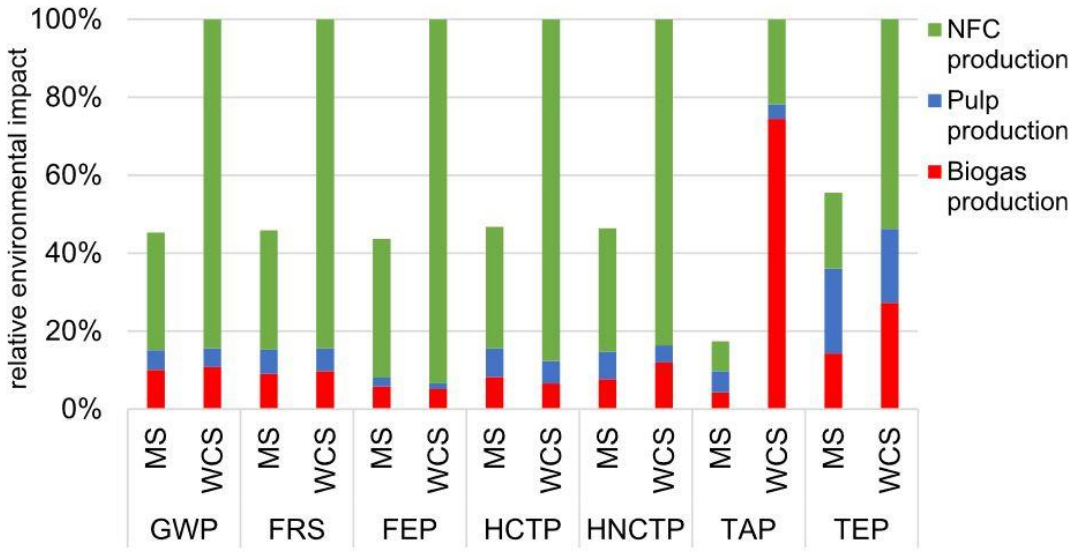

Figure 10 Comparison of environmental impact in the categories: Global warming potential (GWP100), freshwater eutrophication potential (FEP), fossil resource scarcity (FRS), terrestrial acidification potential (TAP), human carcinogenic/non-carcinogenic toxicity potential (HCTP, HNCTP) and terrestrial ecotoxicity potential (TEP)) of the wood chips scenario (WCS) and the manure scenario (MS).[153] (Link in: https://www.sciencedirect.com/science/article/pii/S0301479722006661, accessed 29.09.22, open access under the terms and conditions of the Creative Commons Attribution (CC BY) license)

4.4 Fungal biomass modified lignocellulosic fibres from elephant manure

4.4.1 Chemical composition of fungal biomass modified lignocellulosic fibres

Two white-rot species *P. ostreatus* and *T. versicolor* were used to upcycle elephant manure, which was used as substrate for fungal cultivation. The lignocellulosic fibres contained in elephant manure were modified by the deposition of chitin-β-glucan complex by mycelial growth (**Figure 11**).

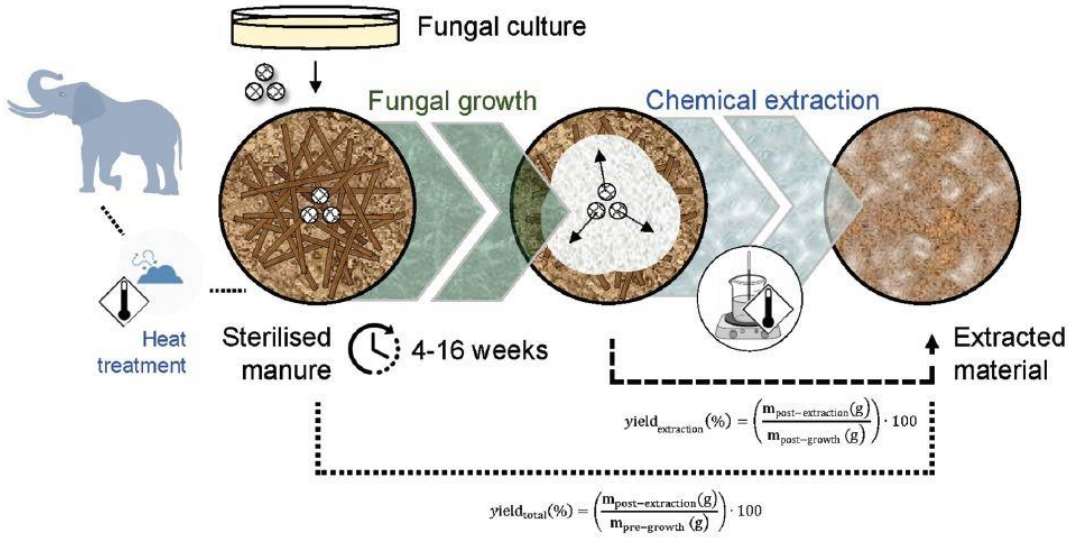

Figure 11 Process for production of fungal biomass modified lignocellulosic fibres extracted from elephant manure on which *P. ostreatus* and *T. versicolor* fungi were cultivated (Link in: https://doi.org/10.1039/D1GC01835C, accessed 11.11.22)[134]

Fungal growth proceeded for 4 to 16 weeks. Both fungal species consumed lignin resulting in a reduction of the lignin content by approx. 7 wt%. *T. versicolor* was associated with a greater reduction (from 21.1 wt% to 14.5 wt%) compared to *P. ostreatus* (23.2 wt% to 16.4 wt%) over a time period of 16 weeks (**Table 6**). With increasing growth time and expanding hyphal network more ligninolytic enzymes were excreted and lignin degradation occurred. Despite both species expressing enzymes that degrade not only lignin but also hemi/cellulose, the glucose content increased as shown by carbohydrate analysis.[154] During fungal growth, chitin-β-glucan complex forms in the fungal cell walls increasing the glucose content in the composite material. Glucose is the building block of glucan. The glucosamine content, indicating the chitin amount, doubled approximately every 4 weeks for the first 12 weeks, before increasing by a factor of 3 from week 12 to week 16. Despite *T. versicolor* having a lower chitin content,[155] the fungal biomass modified lignocellulosic fibres had a higher N content (0.2-0.3 wt%) and glucosamine content (0.1-1.6 wt%) compared to *P. ostreatus* (0.1-0.2 wt% N, 0.1-1.12 wt% glucosamine), which was attributed to the faster growth rate of *T. versicolor* (Table 6). Generation of higher quantities of fungal biomass by *T. versicolor* is also reflected by the extractives content, which increased for both fungal species, by a factor 5 for *T. versicolor* (0.23 wt% to 1.17 wt%) but only by 3.4 for *P. ostreatus* (0.23 wt% to 0.79 wt%).

An overall mass loss was observed for both fungal species from approx. 45 wt% to 18 wt% using *P. ostreatus* and 17 wt% using *T. versicolor*, which was attributed to the conversion of lignocellulosic biomass to water and CO_2 by fungal growth. Additionally, alkaline soluble compounds formed, which are removed during chemical extraction leading to higher purity.

Table 6 Acid insoluble lignin (AIL), extractives and nitrogen (N) content and yield of fungal biomass modified lignocellulosic fibres obtained by *P. ostreatus* and *T. versicolor* growth on elephant manure for 4 to 16 weeks.

Fungal species	Growth time [weeks]	AIL [wt%]	Extractives [wt%]	N [wt%]	$Yield_{composite}$ [wt%]
Reference	0	20.7 ± 0.3	0.23 ± 0.11	0.2	44.8
P. ostreatus	4	23.2 ± 0.2	0.31 ± 0.11	0.3	60.6
	8	19.7 ± 0.6	0.48 ± 0.07	0.1	38.1
	12	16.3 ± 0.5	0.54 ± 0.30	0.2	36.3
	16	16.4 ± 0.3	0.79 ± 0.21	0.2	17.6
T. versicolor	4	21.1 ± 0.4	0.26 ± 0.13	0.2	47.0
	8	15.8 ± 0.2	0.53 ± 0.17	0.2	42.6
	12	19.6 ± 0.3	0.44 ± 0.08	0.2	36.4
	16	14.5 ± 0.5	1.17 ± 0.33	0.3	16.5

4.4.2 Mechanical and surface properties of composite papers

The reinforcing effect of nanofibrous chitin-β-glucan complex and the partial delignification had a significant influence on the mechanical and surface properties of the composite papers prepared from extracted manure and fungal biomass modified lignocellulosic fibres. Papers produced from *P. ostreatus* colonised samples (16 weeks) had the highest tensile strengths of 37 MPa, thus doubling the strength of the reference paper without any fungal growth (16 MPa). After the same growth period papers produced from lignocellulosic fibres modified by *T. versicolor* growth exhibited a tensile strength of only 26 MPa (**Table 7**). This effect was also reflected by the elastic moduli of the papers, which more than doubled using *P. ostreatus* modified fibres (8.0 GPa) compared to the reference material (3.6 GPa). The samples extracted from *T. versicolor* colonised manure had an elastic modulus of 5.8 GPa. The composite papers containing *P. ostreatus* modified lignocellulosic fibres had the highest strain to failure (0.7%). The samples produced from fibres modified by the fungal growth of *T. versicolor* (0.57%) at the same growth time and the reference paper (0.55%) had a 20% lower strain to failure. Lower porosity is most likely the reason for the improved paper tensile properties. The envelope density increased from 0.57 g cm^{-3} for the reference to 0.77 g cm^{-3}

for *P. ostreatus* modified fibres and 0.69 g cm^{-3} for *T. versicolor*, which was accompanied by approx. 14% and 9% reductions in porosity, respectively (*P. ostreatus* 47%, *T. versicolor* 52% and reference 61%). The skeletal density was determined to range between 1.47 to 1.42 g cm^{-3} (**Table 7**).

Table 7 Envelope density (ρ_e), skeletal density (ρ_s), porosity (Φ), tensile strength (σ), elastic modulus (E) and strain to failure (ε) of papers produced from fungal biomass modified lignocellulosic fibres.

Fungal species	Growth [weeks]	ρ_e	ρ_s	Φ	σ	E	ε
		[g cm^{-3}]		[%]	[MPa]	[GPa]	[%]
Reference	0	0.57 ± 0.01	1.47 ± 0.01	61.2	15.5 ± 0.8	3.6 ± 0.3	0.55 ± 0.05
P. ostreatus	4	0.62 ± 0.03	1.42 ± 0.01	56.5	12.7 ± 1.8	3.7 ± 0.5	0.44 ± 0.05
	8	0.57 ± 0.02	1.44 ± 0.01	60.5	14.6 ± 0.7	3.8 ± 0.2	0.50 ± 0.02
	12	0.59 ± 0.02	1.43 ± 0.01	58.6	13.8 ± 2.3	3.7 ± 0.5	0.46 ± 0.06
	16	0.77 ± 0.02	1.46 ± 0.01	46.9	36.6 ± 3.7	8.0 ± 0.6	0.71 ± 0.12
T. versicolor	4	0.57 ± 0.02	1.45 ± 0.01	60.7	13.3 ± 2.2	3.5 ± 0.5	0.49 ± 0.11
	8	0.62 ± 0.03	1.44 ± 0.01	57.0	16.2 ± 3.0	4.3 ± 0.3	0.47 ± 0.08
	12	0.62 ± 0.03	1.45 ± 0.01	57.3	15.3 ± 1.6	4.4 ± 0.4	0.43 ± 0.03
	16	0.69 ± 0.02	1.45 ± 0.01	52.3	26.4 ± 4.9	5.8 ± 0.4	0.57 ± 0.09

The hybridisation effect of the more hydrophobic chitin-β-glucan network bridging the hydrophilic lignocellulose fibres showed a significant influence on the wettability of the composite papers. For composite papers containing only elephant manure-derived lignocellulosic fibres no static water contact angle could be measured. The water droplets imbibed immediately into the paper network. However, it was possible to measure contact angles on papers produced from fungal biomass modified lignocellulosic fibres extracted from manure on which fungi were grown for 4 weeks. These fibres had a glucoseamine content of just 0.1 wt% and an extractives content of 0.31 wt% when using *P. ostreatus* and 0.26 wt% for *T. versicolor*, which was similar to the reference paper (0.23 wt%).

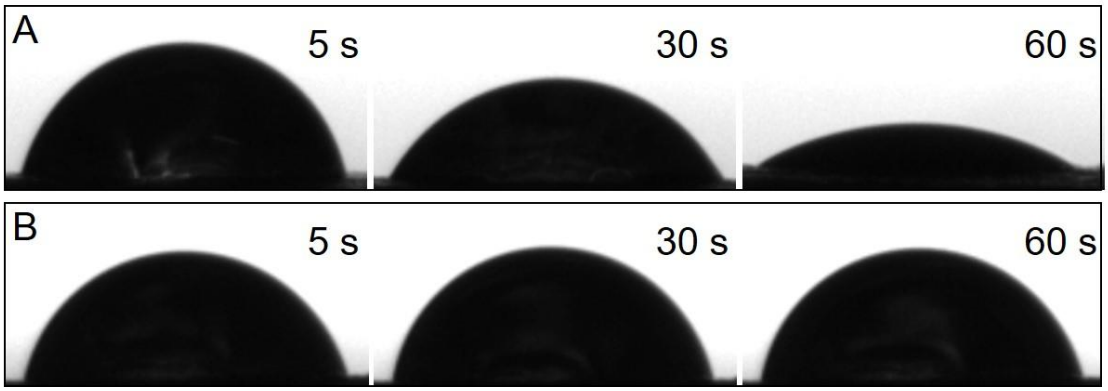

Figure 12 Water droplet after 5 s, 30 s and 60 s resting on composite papers produced from fungal biomass modified lignocellulosic fibres using *T. versicolor* after 4 weeks (A) and 16 weeks (B).

Furthermore, water contact angle measurements revealed a significant increase in the stability of water droplets resting on the paper surface retarding the imbibition of the water droplet into the paper substrate, which was attributed to the increasing chitin content and reduced porosity of the composite papers (**Table 8**). After 4 weeks growth time, the water contact angle measured after 60 s decreased by 16% for *P. ostreatus* and even 43% for *T. versicolor* modified lignocellulosic fibres comprising the composite paper. After 8 weeks growth time, composite papers produced from lignocellulosic fibres modified by *P. ostreatus* exhibited a lower contact angle reduction of 35° compared to *T. versicolor* (30°). After 16 weeks growth period at 60 s exposure time the contact angle decreased by only 5° and 2° for *P. ostreatus* and *T. versicolor*, respectively. By increasing the growth time more chitin-glucan is formed in the growth process leading to a higher surface coverage of the produced composite paper and therefore improved hydrophobicity.

Table 8 Static water contact angle (5 to 60 s water droplet resting time) of papers produced from fungal biomass modified lignocellulosic fibres from elephant manure using *P. ostreatus* and *T. versicolor* with growing times of 4 to 16 weeks. Values for reference paper prepared from lignocellulosic fibres from elephant manure are also provided.

Fungal species	**Growth [weeks]**	**Water contact angle [°]**		
		5 s	**30 s**	**60 s**
Reference	0	0	0	0
P. ostreatus	4	81 ± 7	75 ± 7	68 ± 9
	8	76 ± 7	53 ± 13	41 ± 12
	12	80 ± 8	72 ± 13	63 ± 18
	16	77 ± 6	74 ± 7	72 ± 7
T. versicolor	4	77 ± 7	61 ± 11	44 ± 11
	8	85 ± 7	66 ± 12	55 ± 14
	12	83 ± 9	81 ± 9	76 ± 13
	16	76 ± 10	75 ± 9	74 ± 8

5 Overall Conclusions

The aim of this thesis was to upcycle low grade biomass used as animal feed by manure utilisation into value-added products and to demonstrate the possibility of a novel manure management system. Elephant and (horse manure) was utilised as model system for the production of biogas, a fertilizer precursor and nanocellulose. Manure was used as substrate for biogas production over a time period of 40 days. Samples of the fermentation residue were taken after different AD times. Manure and fermentation residue were chemically treated to extract (ligno)cellulosic fibres, which were subsequently defibrillated into NFC using a disc mill. I could show that the energy required for NFC production from manure was at the lower range of common NFC production processes reported from high grade woody biomass, which highlights the benefits of manure pulp for NFC production. The produced NFC was used to manufacture nanocellulose papers, which were characterised with regards to their chemical and mechanical properties. From elephant manure biogas and methane could be produced in high yields. During the process of anaerobic digestion lignin was accumulated in the fibre material. The NFC papers produced from fibrils recovered from elephant manure and fermentation residue exhibited mechanical properties comparable to or even outperforming conventional NFC papers produced from high grade biomass, such as wood pulp. Additionally, the LCA performed proved the superiority of the developed process over conventional biogas and pulp production. It was shown that current manure management systems can be optimised to generate not only a one or two value-added products (biogas and/or farm fertilizer), but a cascade of products by combining multiple processes to upcycle manure to realise its full potential. Furthermore, the developed herbivore manure utilisation process also highlights the potential to produce ligno/cellulosic fibres from low grade biomass, such as grasses and shrubs, collected and digested by animals that extract their livelihood from this feed.

White-rot fungal growth on elephant manure substrate was explored to partially remove lignin from the fibre substrate and to modify the fibres by deposition of fungal cell wall structural polymers. This biological modification generated a composite material by integration of nanosized chitin-glucan complex fibres and micron-sized lignocellulosic fibres present in

elephant manure. The mechanical properties of the composite papers improved with increasing amount of deposited fungal cell wall polymers, i.e. increasing with increasing growth time, while the paper's hydrophobicity significantly improved; the water contact angles increased and the imbibition of water droplets into the paper substrate was significantly decreased.

Improving the water resistance of papers produced from lignocellulosic fibres can also be achieved using anaerobic digestion to up-concentrate lignin in LCNFs. The fibre component analysis showed that using AD, fibres with a tuneable cellulose to lignin ratio can be obtained. Horse manure was treated by anaerobic digestion in a fermenter to generate high biogas yields and the fermentation residue was used for the production of LCNFs with lignin contents of up to 29 wt%. high lignin contents allowed for significantly reduction of the water retention capability of nanopapers made from such fibrils.

Overall, it was possible to show that manure is a suitable raw material for the production of a diverse product range, biogas, fertilizer precursor and high-quality LCNF/NFC, enabling the full exploitation of this feedstock while minimising the environmental impact of traditional manure utilisation methods.

6 Outlook

The work presented lays the foundation for future research focusing on the valorisation of manure, an omnipresent agricultural waste material, into upcycled products. It was shown that biogas, NFC/LNFC and a fertilizer precursor can be obtained by a strategic arrangement of chemical and/or biological manure processing unit operations. By variation of treatment parameters, such as duration and chemical agents, materials with tuneable properties, such as cellulose/lignin or cellulose/chitin ratios and thus mechanical and surface properties can be obtained.

The results presented in my thesis do emphasise the challenge of valorisation of agricultural by-products due to heterogeneity of resources, changes in available volume over time and regional availability. To implement a successful manure management system fulfilling the

regulatory and legislative requirement utilising a greater volume of raw manure would allow for mitigation of heterogeneity issues of the substrate. By adjusting manure composition, for instance by mixing with other agricultural waste streams, it would be possible to provide an optimal composition for all upcycling process steps. Two manure types, i.e. horse and elephant, were selected as model systems demonstrating the feasibility of the process because of their high content of lignocellulosic raw fibres. However, the high abundancy of cattle, poultry and swine livestock renders these manure types interesting as substrate for a circular manure management system and will open valorisation routes for these manure types. The process will have to be adapted because of their different composition with regards to lignocellulosic fibre and nutrients content. The utilisation of cattle, poultry and swine manure in the developed manure upcycling process will have to be tested. Furthermore, the fertilizer potential of the resulting fertilizer precursor is to be assessed.

I also showed that herbivore manure is suitable substrate for mushroom farming. This potential could be further explored and should allow for the reduction of the high costs associated with common cultivation substrates, such as beech wood chips for the production of (edible) mushrooms used for cosmetic/textile applications.[156] The utilisation of a manure substrate for mushroom farming will not only result in fruiting bodies but also partially digested substrate (manure or wood), which can be used as resource as fertilizer or for the pulp and paper production.

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8 Publications

Publication I

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Research Article

Excellence in Excrements: Upcycling of Herbivore Manure into Nanocellulose and Biogas

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ABSTRACT: The demand for animal products has significantly increased over the past decades as a result of the growing population and the heightened standards of living. Increased livestock farming does not only yield desired products but also significant quantities of wastes, particularly manure whose storage and application are being monitored with a tightening network of regulations. The problem is that manure is considered merely as a substrate for biogas production or as a fertilizer, whereas the substantial portion of fibers residing in herbivore manure has remained underutilized. Here, we propose a manure management system, in which not only biogas and fertilizer precursors but also high-value materials in the form of (nano)cellulose are produced. We show that high biogas yields can be achieved for elephant manure and the remaining substrate enables effortless isolation of cellulose nanofibers, leading to a significant reduction of the environmental impact compared with traditional systems based on wood.

KEYWORDS: *elephant manure, biogas, nanocellulose, manure management, tensile properties*

INTRODUCTION

Climate change has bestowed unforeseen engineering challenges upon the modern society. The most pressing of those challenges have been connected to meat and dairy production systems. In 2018, 436 Mt CO_2 equiv of greenhouse gases (GHG) were emitted by agricultural operations in the EU-27 alone, thus coming only second after the energy sector,[1] with manure being one of the main emitters of non-CO_2 GHG within agriculture, particularly rife in methane and nitrous oxides.[2] Additionally, this nutrient-rich material, riddled with pathogens, also adds to the eutrophication and contamination of soil and surface water if its release into the environment is uncontrolled.[3,4] What is often overlooked is that herbivore manure encompasses useful fibrous material because of its notoriously inefficient digestion. Depending strongly on the feed and digestion system of the animal, herbivore's manure can still contain 50−80% intact lignocellulosic material.[5,6] While livestock manure is already utilized as a fertilizer and in biogas production, it contains a lot of fibers that remain idle within these applications.[7] Indeed, anaerobic digestion (AD) is able to convert much of the compounds, like proteins and small-molecular carbohydrates, into CH_4, which can be used as a biofuel or source of electricity in a combined heat and power plant. Nevertheless, recalcitrant polymers, such as cellulose, are generally not converted completely and remain in the fermentation residue. In addition, there is simply an over-abundance of manure in intensive livestock farming areas that are situated away from crop farming regions. For example, in the Netherlands, 17.9 Mt of the total 68.6 Mt animal manure produced in 2011 had to be exported, resulting in additional environmental burden and costs.[8]

Here, we propose a solution to elevate the use of manure into a completely new level as a source of high-value-added materials. Although much of the fibers pass the digestive system seemingly unchanged, their ultrastructural morphology has been markedly changed, resulting in an altogether more refined structure that better enables the disintegration of the fibers into nanocellulose. This apparently trivial procedure is a major engineering challenge in modern materials science concerned with renewable materials. For the best part of the current century, cellulose nanofibers (NFCs) have been touted as a promising sustainable alternative in, e.g., composite manufacturing,[9,10] membrane technology,[11,12] and medical applications,[13,14] but the high price and/or energy consumption of their preparation has impeded the genuine

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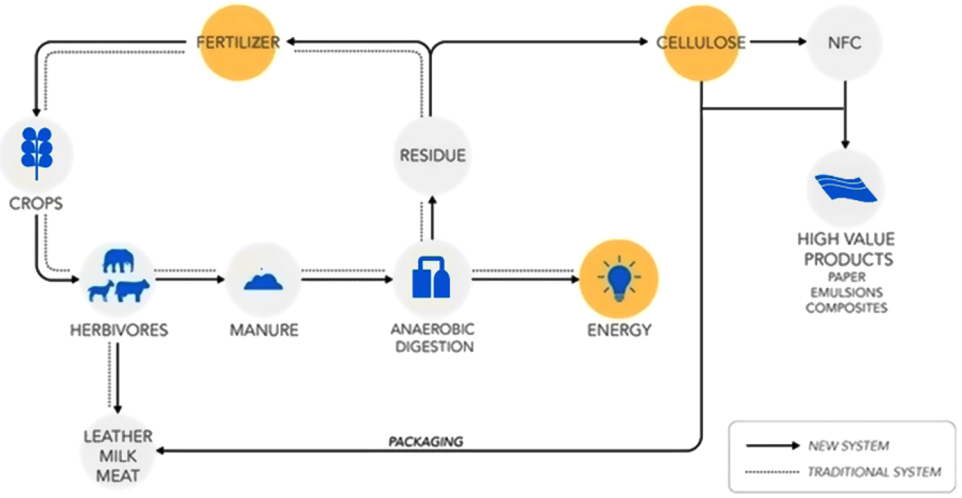

Figure 1. Graphical description of the envisioned production chain of value-added products from animal manure: conventional use of manure (traditional system, dotted line) and new production system (new system, solid line).

industrial triumph of NFCs so far.[15] According to Global Market Insights, by 2026, the nanocellulose business is anticipated to reach $418 million, but to fulfill the potential, the production costs must be decreased by new solutions.[16]

In this study, we are set to solve the very different problems of underutilized manure and energy consumption in NFC production. As a model system, we have used elephant manure. It has one of the highest cellulose contents among herbivore manure, as they only digest 30−40% of their feed, and comprises approximately 40 wt % cellulose, 23 wt % lignin, and 10 wt % hemicellulose. The elephant's digestive tract is representative of all large animals with monogastric digestion system.[6,17] For example, horse manure contains a similar amount of cellulose (20−30%), and therefore, the findings reported here are also applicable to horse manure.[18] It is estimated that 40−70 Mio tons per year is produced in Europe alone.[19] We show how animal manure as well as fermentation residues can be utilized to their full potential by generating products, such as NFCs, biogas, and fertilizer precursors (Figure 1). The quality of NFC was ascertained by characterizing the physicochemical and mechanical properties of cellulose nanopapers prepared thereof. The envisioned production chain not only upcycles manure and alleviates NFC production but also outsources the plant harvesting and processing to herbivores, thus reducing the effort and environmental costs of cutting down trees as well as pulping, bleaching, and defibrillation operations, which are only set to grow in demand in a modern society hungry for more renewable materials solutions. The environmental impact of the proposed production cycle was evaluated by lifecycle assessment and juxtaposed with the traditional paper production from wood and will be reported in Krexner et al.[20]

MATERIALS AND METHOD

Materials. Fresh elephant manure was provided by the Vienna Zoo "Tiergarten Schönbrunn". Different batches of manure (ca. 10 kg each) were collected in April, June (2018), and January (2019). If not mentioned otherwise, the discussed results were generated with material collected in April 2018. Sodium hypochlorite (NaOCl, 14% active Cl, W. Neuber's Enkel, Austria), sodium hydroxide (NaOH, 99.6%, Sigma-Aldrich), and sulfuric acid (95%, Sigma-Aldrich) were used as received. Neutral detergent solution concentrate, acid detergent solution powder, and triethylene glycol were purchased from Ankom Technology (Macedon). Decahydronaphthalene was supplied by Merck (Darmstadt, Germany). Microcrystalline cellulose was purchased from Sigma-Aldrich. As a reference, birch kraft pulp (KP) was used. For all experiments, distilled water was used. The inoculum for biogas experiments consisted of two inocula from a biogas plant located in Margarethen am Moos and Ziersdorf (Austria), respectively.

Specific Methane Yield and Degradation Kinetic Studies of Elephant Manure. Anaerobic digestion trials of elephant manure were performed in triplicate according to standard VDI 4630 (VDI—The Association of German Engineers 2016). Eudiometer batch fermenters (250 mL) were filled with 200 mL of inoculum in a 3:1 ratio based on volatile solid (VS) content and stirred continuously at 37.5 °C (mesophilic conditions) over the course of 40 days. Microcrystalline cellulose was used as a control. The methane and biogas production were monitored on a daily basis. Gas volumes are reported at 273.15 K and 101.33 kPa per kilogram of volatile solids (L_N kg^{-1} VS). The methane (CH_4) and carbon dioxide (CO_2) contents were analyzed with a portable gas analyzer (Dräger X-am 7000, Dräger, Lübeck, Germany), calibrated weekly with a gas standard consisting of 33% CH_4 and 33% CO_2 (Messer, Gumpoldskirchen, Austria).

Investigation of the degradation kinetics of structural compounds of elephant manure was performed following the procedure described previously.[21] Samples were taken after 5, 10, 20, 30, and 40 days of AD, respectively, and washed thoroughly with deionized water. The samples were dried at 50 °C until constant weight.

Chemical Analysis of Manure and Fermentation Residue. Elephant manure and fermentation residues were investigated with regard to their water, cellulose, hemicellulose, and acid detergent lignin (ADL) contents using the method of Naumann and Bassler.[22,23] The material was first ground using a cryomill (CryoMill, Retsch, Haan, Germany) at a frequency of 28 Hz for 14 min after precooling with liquid nitrogen at a frequency of 5 Hz for 2 min. The water content was evaluated using a Karl Fischer titrator (Mettler Toledo V20, Columbus, Ohio). To analyze the influence of anaerobic digestion on the structural fiber components, degradation yield ($yield_{degradation}$) was calculated based on the biomass before biogas production and the corresponding fiber contents of cellulose, hemicellulose, and ADL after days 0, 5, 10, 20, 30, and 40 of AD (eq 1).

$$\text{yield}_{\text{degradation}}\ (\%) = \frac{\text{mass biogas residue (g)} \cdot \text{fiber content (\%)}}{\text{mass before biogas production (g)}} \quad (1)$$

Elemental analysis was performed using an EA 3000 CHNS-O elemental analyzer (Eurovector, Italy) equipped with a high-

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temperature pyrolysis furnace (HT, Hekatech, Germany). Fourier transform infrared (FT-IR) spectra were recorded on a spectrometer (Carry 630, Agilent Technology, Austria).

Extraction of Cellulose Fibers from Elephant Manure and Biogas Residue. Dried manure or biogas residue, respectively, were washed over a 300 μm mesh sieve, and the sand was removed by sedimentation. Alkaline treatment (20 g manure/AD residue L^{-1}) was conducted in 0.1 M NaOH for 2 h at 80 °C.

The liquid residue of the alkaline treatment was neutralized and dried. $Yield_{extract}$ was calculated based on the received dry mass in the extract and the mass before biogas production in dry mass (eq 2)

$$\text{yield}_{\text{extract}}\,(\%) = \frac{\text{received material of alkaline extraction (g)}}{\text{mass before biogas production (g)}} \times 100\% \quad (2)$$

The residue was rinsed with water over a 75 μm mesh sieve. Bleaching was performed using 4 g of dry matter (DM) of alkaline-pretreated manure stirred overnight (17 h) in 660 mL of a 0.4 M NaOCl solution. The suspension was washed over a 75 μm mesh sieve, dewatered, and stored at 4 °C.

The total yield ($yield_{total}$) was calculated based on the mass of the lignocellulosic material received after the extraction process over the mass before biogas production in dry mass using eq 3.

$$\text{yield}_{\text{total}}\,(\%) = \frac{\text{material after extraction (g)}}{\text{mass before biogas production (g)}} \times 100\% \quad (3)$$

Nanocellulose Production. To produce nanocellulose, the extracted cellulose fibers (50 g DM as 3.5% suspension in water) were blended for 4 min at 1000 rpm (JB 3060, Braun) and subsequently passed through a disk mill (Granomat JP 150, Fuchs, Switzerland). Extracted fibers from elephant manure without prior biogas production were used to investigate the influence of grinding cycles on the material. The material was ground 1, 2, 5, 7, and 10 times, respectively. This procedure was done for two different batches. Extracted fibers from biogas residue were ground 10 times. The resulting gels were dewatered over a textile cloth to a consistency of approximately 2 wt % and stored at 4 °C. As a reference, nanocellulose was produced from kraft birch pulp by grinding 10 times. The energy consumption was monitored using an energy logger (Voltcraft 4000) and a high-voltage meter (Swissnox SX-3M).

(NFC)Paper Production. For papers with a grammage of 100 g m^{-2}, 1.22 g (DM) extracted fibers were blended for 3 min at 1000 rpm with 300 mL of water (JB 3060, Braun) and filtered over a Büchner filter funnel (VitraPOR, 100 mL, Por. 3, Robu, Hattert, Germany) lined with a filter paper (VWR 413, 125 mm in diameter). The filter cake was pressed between two sheets of blotting and one sheet of baking paper in a heated hydraulic press (model 412 6CE, Carver) two times at 20 °C at a 20 kN force and subsequently at 120 °C for 15 min applying a 20 kN force.

Tensile Properties of (NFC)Papers. For the determination of the tensile properties of the produced papers dog bone-shaped specimens (shape after type 1BA, EN ISO 527-2) with 5 mm parallel width and 75 mm overall length (Zwick ZCP 020 Manual Cutting Press, Zwick, Ulm, Germany) were cut. Tensile tests were performed using a universal test frame (Model 5969, Dual Column Universal Testing System, Instron, Darmstadt, Germany) equipped with a 1 kN load cell and a noncontact video extensometer (Gig ProE, iMETRIUM, Bristol, U.K.) at a temperature of 26 °C and a relative humidity of 50% with a testing velocity of 1 mm min^{-1}. The gauge length was set to 25 mm. The tensile strength (σ) was calculated from the maximum load and the cross-sectional area of the specimen. Young's modulus (E) was analyzed in the linear elastic region of the stress−strain curve as secant between strength values separated by 0.2% strain (ε).

Morphology of (NFC)Papers. Scanning electron microscopy (SEM) images of the (NFC)papers were obtained with a Zeiss Supra 55VP at an accelerating voltage of 2 kV and a working distance of 7.2 mm. The samples were coated with a 10 nm gold layer (Leica scd 050/EM QSG 100) at 60 mA for 67 s. The fiber diameters were determined with the operating software for field emission scanning electron microscope SmartSEM V05.04.

RESULTS AND DISCUSSION

Elephant Manure as Feedstock for Biogas Production. The collected and dried elephant manure had cellulose, hemicellulose, and ADL degradation yields of 31, 28, and 15 wt % of dry mass, respectively (see Supporting Information Table S1). The high share of lignocellulosic material of 74% highlights the inefficient digestive system of the elephants and signifies the suitability of manure as a raw material for the proposed system. With an ash content of 5 wt % representing the inorganic content, the organic residue can be estimated to be 8 wt %. Thus, it had a similar cellulose content to wheat straw (35−39 wt %), approaching that of wood (45−50 wt %) and comparable to cow manure (30%).[24,25] The difference being that elephants are monogastric herbivores, while cattle are ruminants. With straw and hay being the main feed of elephants kept in captivity, it signifies that the cellulose yield was not compromised by the elephant's digestion system but had the advantage of collection as well as mechanical and enzymatical pretreatment by the animal.[26] Manures collected at different times (April (0-1), June (0-2), and January (0-3)) varied in their composition (see Supporting Information Table S1). The raw material 0-2 collected in June exhibited the highest cellulose degradation yield (57%), and the material 0-3 collected in January had the highest ADL degradation yield (29%). This variation occurs because of differences in seasonal food supply for the elephants.

The volatile solid content (VS) corresponds to the organic dry matter in the material without inorganic matter and gives an orientation of how much organic material can be converted into biogas. With 89%, a high content of organic biomass is convertible into methane, indicating that elephant manure is a promising feedstock for the anaerobic digestion process. Elephant manure had a VS comparable to wheat straw (83−88%)[27,28] and other agricultural biomass (maize silage 96%,[29] grass silage 86%[29]). The C/N ratio (46:1; see Supporting Information Table S2) on the other hand is a crucial parameter for the process stability during AD. A low C/N ratio leads to ammonia intoxication of the fermentation system and inhibits the AD process, whereby a too high ratio microbial biomass cannot be maintained. The optimal C/N ratio for biogas production is widely discussed in the literature and is found to be optimal at approximately 30:1, but no ammonia intoxication is expected at a C/N ratio at 46:1.[30]

During the first 10 days of anaerobic digestion, the biogas and methane yields were 66% of the total biogas (262 L_N kg^{-1} VS) and 64% (156 L_N kg^{-1} VS) of total methane. After 40 days of fermentation, the final specific biogas yield was 399 L_N kg^{-1} VS with a methane content of 61% (244 L_N kg^{-1} VS) (Figure 2). A previous study reported a similar amount of methane produced from zoo animal manure in 12 days.[31] Using hay as biogas substrate resulted in almost the same methane yield (243 L_N kg^{-1} VS) but a lower methane concentration (58%) at an overall biogas yield slightly higher than in our study (420 L_N kg^{-1} VS).[32] Most of the biogas and methane is produced during the first 10 days of AD by preferential conversion of hemicellulose and cellulose (Figure 2);[33] their yield decreased by 55 and 54%, respectively. Between day 10 and day 40 in the fermenter, the yield of both structural polymers decreased further by approximately 30%. The ADL yield remained almost

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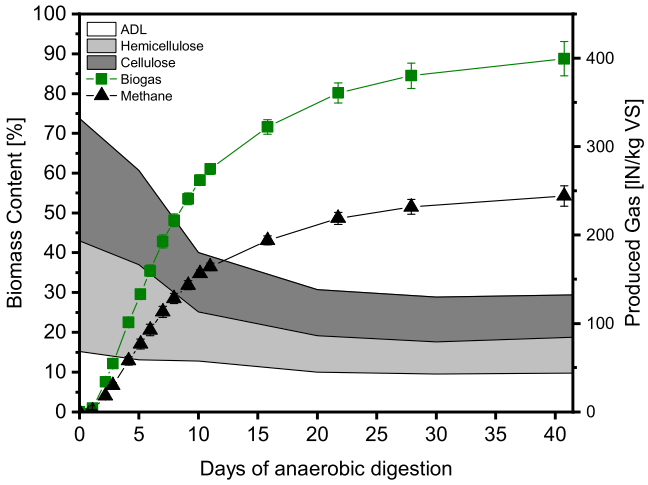

Figure 2. Biogas (green square) and methane (black triangle) yields (L_N kg^{-1} VS) as a function of fermentation time using elephant manure as feedstock. ADL (white), hemicellulose (gray), and cellulose (dark gray) contents in manure (day 0) and the digestate after 5−40 days of anaerobic digestion, respectively.

constant, showing that lignin was converted into biogas to a much smaller extent and hence up-concentrated in the material.[34]

An alkaline treatment was performed using 0.1 M NaOH to separate the fibrous material from nitrogen-containing compounds, such as proteins, keratinized tissue, and dead cells, which can potentially be used as a fertilizer and originated from the elephant's digestion process. The liquid residue after alkaline treatment was neutralized and dried to be considered as a fertilizer precursor. $Yield_{extract}$ ranged here from 25% for material without any prior anaerobic digestion and decreased to 11% for AD of 40 days. All liquid extracts after AD contained between 20 and 28% carbon and <1% nitrogen, which is in accordance with the fertilizer regulation (BGBl. II Nr. 100/2004) (see Supporting Information Table S4).[35] Optimization regarding nitrogen, phosphorus, and mineral content to meet the soils need would be possible through additional purification and up-concentration processes.

To come up with white cellulose pulp, a bleaching treatment was conducted. $Yield_{total}$ of lignocellulosic fibers after alkaline and bleaching treatment of elephant manure without prior anaerobic digestion was 41% (Table 1) and represents only the yield after the chemical treatment. AD prior to chemical extraction resulted in a yield reduction (21% for 5 days AD to 12% for 20 and 30 days of AD). Wood as raw material using the established Kraft process has yields of 46−50%, which seems significantly higher.[36,37] But it needs to be pointed out that already biogas in high yields is produced from the fermentation residue and that this is a low-grade biomass compared with wood. Cellulose could be extracted from cow manure with a yield of 11% without prior AD, which is similar to elephant manure after 20−40 days of anaerobic digestion.[38]

Mechanical Properties of Papers Prepared from Biogas Residues. The tensile properties of papers produced from extracted fibers were evaluated to assess the impact of biogas production and hence the altered composition of the raw material (Table 1, representative stress−strain curves in Supporting Information Figure S1) and degradation of the fibrous material. Papers produced from lignocellulosic fibers extracted from biogas residue (5 days AD) had even higher tensile strength (74 MPa), Young's modulus (11 GPa), and strain to failure (1.4%) than the reference paper produced from elephant manure extract without prior anaerobic digestion (60 MPa). The tensile strength of papers produced from lignocellulosic fibers extracted from unfermented manure or fermentation residue after 5 and 10 days outperformed ordinary copy paper (20−50 MPa) with the advantage of utilizing more sustainable raw material than wood and that a further value-added product (biogas) was also obtained in this process.[39,40]

For papers obtained from fibers that were retrieved after longer fermentation times (20−40 days), a significant decrease in tensile strength (~30 MPa), modulus (~6 GPa), and strain to failure (0.6−0.7%) was observed. These lower tensile properties of the produced papers can be attributed to the varying composition of the material during AD: With increasing fermentation time, lignin is up-concentrated, while the hemicellulose content decreases, but the molecular weight of the extracted lignocellulosic fibers did not follow a specific trend and is therefore more influenced on the variety of the biological material than influenced by the anaerobic digestion process (see Supporting Information Table S3). Lignin, due to its chemical structure, including hydrophobic aromatic rings, weakens particularly the hydrogen-bonding network between the cellulose fibrils and thus the paper strength decreases.[41] After extended AD (20, 30, and 40 days), between 8 and 10% lignin remains in the extracted fibers (Table 1). Only a minor increase in nitrogen content from <0.05 to 0.08 wt % indicates remaining compounds after the chemical extraction (see Supporting Information Table S3).

SEM images of the produced papers showed separated cellulose fibers with diameters of 13 μm with a high density of connection points between the fibers. The average fiber diameter was not affected by increasing the fermentation time. However, it appears that the fibers were embedded in a matrix

Table 1. Tensile Strength (σ, MPa), Elastic Modulus (E, GPa), Strain to Failure (ε, %), Fiber Diameter (μm), and ADL Content (wt %) of the Produced Paper of Cellulose Extracted from Fermentation Residue of Elephant Manure after 0−40 Days of Anaerobic Digestion[a]

days of AD	σ [MPa]	E [GPa]	ε [%]	$yield_{total}$ [%]	fiber diameter [μm]	ADL [wt %]
0	60.4 ± 4.9	9.1 ± 0.7	1.22 ± 0.12	41	11 ± 5	2.3 ± 0.4
5	74.0 ± 2.3	11.1 ± 1.2	1.37 ± 0.11	21	13 ± 8	4.3 ± 0.03
10	66.2 ± 6.8	9.7 ± 0.6	0.92 ± 0.08	17	13 ± 8	5.2 ± 0.5
20	32.2 ± 2.3	6.2 ± 0.8	0.68 ± 0.04	12	15 ± 10	8.3 ± 0.3
30	31.2 ± 8.4	5.7 ± 0.8	0.70 ± 0.13	12	13 ± 8	8.9 ± 0.2
40	27.4 ± 3.4	5.7 ± 0.3	0.58 ± 0.07	12	13 ± 7	10.4 ± 1.4

[a]$Yield_{total}$ (%) of received lignocellulosic fibers after biogas production and extraction.

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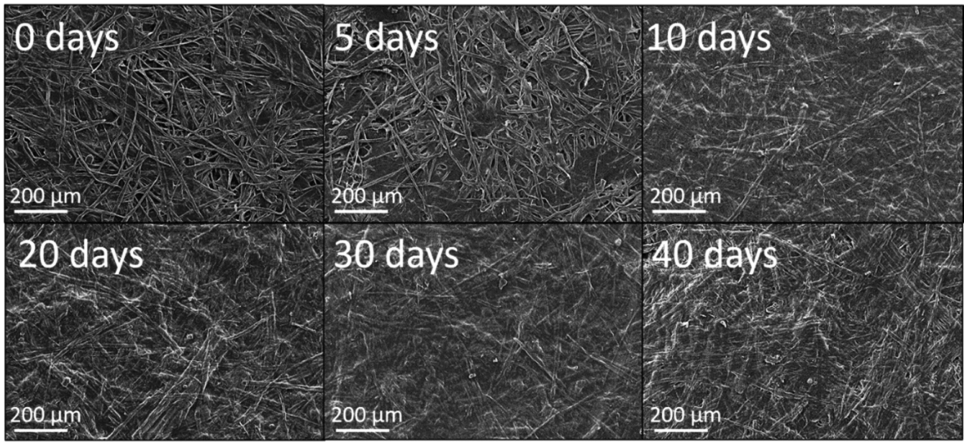

Figure 3. SEM micrographs (100× magnification) detailing the surface morphology of the produced papers using lignocellulosic microfibers extracted from elephant manure (0 days) and fermentation residues.

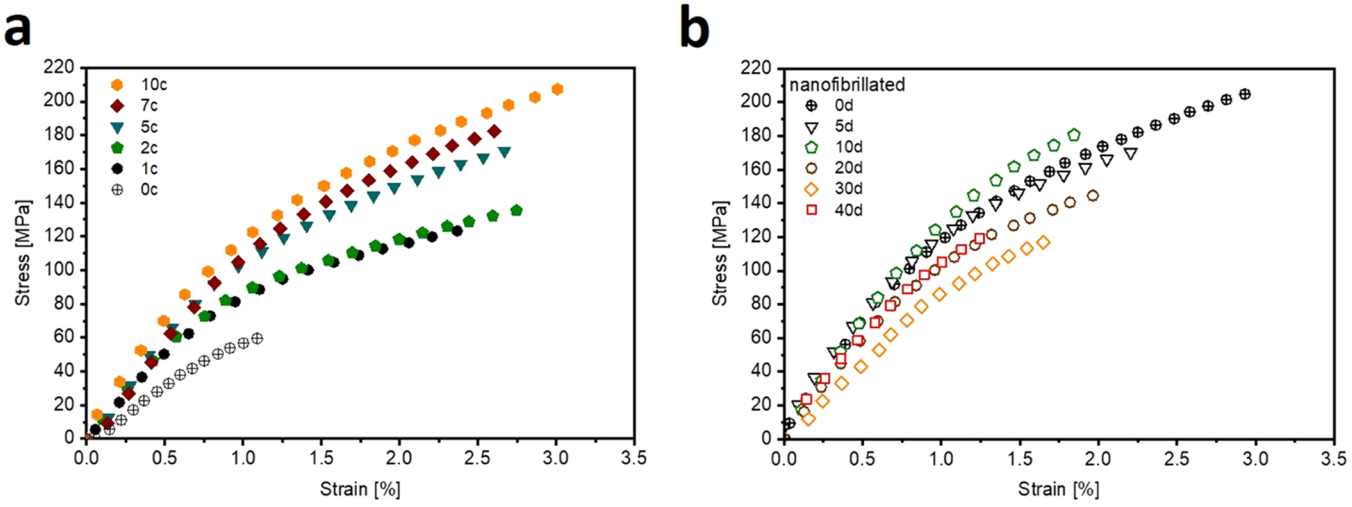

Figure 4. (a) Representative stress−strain curves of nanopapers manufactured using NFC produced by grinding extracted lignocellulose fibers from elephant manure without prior anaerobic digestion after 1 (1c, black dot), 2 (2c, green triangle), 5 (5c, blue triangle), 7 (7c, red diamond), and 10 (10c, orange hexagon) grinding cycles, as well as reference paper produced from elephant manure cellulose fibers (0c, crossed dot). (b) Representative stress−strain curves of nanopapers produced by grinding lignocellulosic fibers extracted from elephant manure 10 times (crossed circle) and from fermentation residue after 5 (black triangle), 10 (green pentagon), 20 (brown circle), 30 (orange diamond), and 40 (red square) days of anaerobic digestion (nanofibrillated).

of lignin, causing a reduction in connectivity between the fibers (Figure 3).

Nanofibrillation for Improved Paper Tensile Properties. Already after one grinding cycle (0.3 kWh kg^{-1}), the tensile strength of the papers increased to 126 MPa thus doubling compared to microfiber papers (64 MPa) (Figure 4a and Table 2). The modulus increased by 173% (from 7.1 to 12.3 GPa), and the strain to failure increased by approximately 50% (2.1%). Fiber agglomerates with diameters of 7−0.5 μm were present in the material, but the majority of fibers was already in the nanometer range (see Supporting Information Table S5). After 10 grinding cycles (5.2 kWh kg^{-1} total energy input), the tensile strength improved further, reaching ∼200 MPa. The increased tensile strength with increasing energy input can be attributed to the higher homogeneity of the material and decreased fiber diameter, leading to increased bonding between the fibers.[42] Reference paper fabricated from Kraft pulp had significantly lower tensile strengths of 16, 30, and 57 MPa after one, two, and five grinding cycles, respectively (see Supporting Information Table S5), which might be associated with larger fiber diameters that were still in the micrometer range after the two first grinding cycles (19 and 15 μm) (see Supporting Information Table S5). Nanometer-scale fibers were only obtained after the fifth grinding cycle, but they were still 50% bigger than fibrils produced by grinding of elephant manure fibers.

These NFC papers produced highlight the material's potential of elephant manure even without the previous production of methane from the material. The tensile properties are on par or even outperformed nanocellulose papers produced from high-grade biomass, such as wood as well as NFC papers from agricultural waste reported in the literature.[43−46]

The impact of variations in feed and thus seasonal influences, in particular the significantly higher ADL contents of 22 and 34 wt %, were confirmed by producing nanopapers from elephant manure collected in June and January exhibiting tensile strengths of 130 and 96 MPa, respectively (Table 2, Supporting Information Table S5).[17] After 10 grinding cycles, nanofibrils derived from the material collected in June and

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Table 2. Tensile Properties: Tensile Strength (σ, MPa), Elastic Modulus (E, GPa), and Strain to Failure (ε, %) of NFC Papers as well as Average Fiber Diameter (nm) of Lignocellulosic Fibers Extracted from Elephant Manure with and without Prior AD[a]

days of AD	cycles	σ [MPa]	E [GPa]	ε [%]	fiber diameter [nm]
0	1	123 ± 6	12.3 ± 0.8	2.1 ± 0.3	36 ± 17
0	2	142 ± 11	12.6 ± 0.9	3.2 ± 0.4	32 ± 24
0	5	171 ± 23	11.7 ± 0.7	2.9 ± 0.5	27 ± 6
0	7	181 ± 16	11.9 ± 1.0	3.2 ± 0.6	33 ± 14
0	10	196 ± 12	11.6 ± 1.2	3.6 ± 0.6	22 ± 4
5	10	173 ± 7	14.5 ± 0.6	2.7 ± 0.6	22 ± 7
10	10	185 ± 7	14.2 ± 1.0	2.2 ± 0.4	32 ± 8
20	10	144 ± 7	13.5 ± 0.3	1.9 ± 0.2	27 ± 6
30	10	115 ± 5	13.3 ± 1.3	1.7 ± 0.2	27 ± 6
40	10	125 ± 9	12.9 ± 0.6	1.6 ± 0.3	31 ± 6
KP	10	102 ± 6	9.7 ± 3.4	3.7 ± 0.5	43 ± 13

[a]Values for the reference material from Kraft pulp (KP) are provided as well.

January had fiber diameters of 40 and 44 nm, respectively, which is ~50% higher than those produced from raw material collected in April.

Papers produced from nanofibrillated lignocellulosic fibers extracted from fermentation residue after 10 days of AD had a tensile strength of 185 MPa and a modulus of 14.2 GPa. The tensile strength was slightly lower, but the modulus was higher than that for nanopapers produced from fibers extracted from unfermented manure, with the major benefit that additionally biogas was produced. The reduction of the paper tensile strength from nonground lignocellulosic fibers extracted after 0−40 days of AD was 57%, whereas for the NFC papers, it was only 47%. The reduction of tensile strength of the papers produced using nonfibrillated fibers was caused by the up-concentration of lignin on the fibers (Figure 4b), which was partially mitigated by the nanofibrillation process. Papers produced from ground lignocellulosic material obtained after 40 days of fermentation had a tensile strength of 125 MPa, a modulus of 13 GPa, and a strain to failure (1.6%), which was still higher than the tensile properties of nanopapers from Kraft pulp. Nanocellulose papers from Norway spruce with 4 and 14% residual lignin had similar tensile strengths of 156 and 125 MPa, respectively.[47] Nanocellulose papers produced from *Miscanthus* straw, which had been anaerobically digested for 4 months, had a tensile strength of 155 MPa.[48] This is comparable to NFC papers from elephant manure after 20 days of anaerobic digestion, although the cellulose from *Miscanthus* straw was extracted with a harsher chemical procedure (extraction with organic solvents such as hexane and dichloromethane followed by multiple bleaching steps using $NaClO_2$).

CONCLUSIONS

Elephant manure was used as a model system to demonstrate the usefulness of livestock wastes as a raw material for the simultaneous production of biogas and (nanofibrillated) lignocellulosic fibers to yield high-performance (nano)papers and fertilizer precursors. After 10 days of AD, a biogas yield of 262 L_N kg^{-1} VS with 64% methane content was obtained, while (nano)papers produced from extracted lignocellulosic material had tensile properties (185 MPa at 100 g m^{-2}) similar to or exceeding those of papers produced from conventional raw materials, such as wood pulp. This shows that a multiple-output system for the utilization of animal manure as a resource can improve the sustainability of livestock waste management systems by reducing waste and energy consumption while simultaneously yielding high-quality products.

ASSOCIATED CONTENT

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acssuschemeng.1c05175.

Additional supporting results, such as elemental composition and degradation yield of fermentation residue, elemental composition of extracted fibrous material from fermentation residue and elephant manure, elemental composition of neutralized dried residue after alkaline treatment, and mechanical properties of the produced paper of cellulose extracted from elephant manure and fermentation residue (PDF)

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Notes

The authors declare no competing financial interest.

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Excellence in excrements: Upcycling of herbivore manure into nanocellulose and biogas

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Supplementary Tables

Table S1 Degradation yield of hemicellulose, cellulose and lignin content (wt.%) of the biogas digestate after 5 to 40 days of anaerobic digestion and elephant manure collected at different month (April (0-1), June (0-2), January (0-3))

Days of anaerobic digestion	Cellulose [wt.%]	Hemicellulose [wt.%]	Lignin [wt.%]
0-1	30.7	27.7	15.2
0-2	57.1	5.9	19.1
0-3	39.6	9.3	28.6
5	23.7	24.0	13.0
10	15.0	12.4	12.8
20	11.5	9.2	10.0
30	9.5	8.1	9.5
40	9.8	9.0	9.8

Table S2 Elemental composition (C, H, N, O in wt.%) of elephant manure collected in April (0-1), June (0-2) and January (0-3) and fermentation residue after 0 to 40 days of anaerobic digestion.

Days of Anaerobic digestion	C [wt.%]	H [wt.%]	N [wt.%]	O [wt.%]
0-1	46.0	6.0	1.0	43.4
0-2	44.4	5.8	0.7	43.7
0-3	40.1	5.5	1.5	43.8
5	43.8	5.7	0.8	41.4
10	45.6	5.8	0.8	39.5
20	44.3	5.8	1.0	39.9
30	45.6	5.9	1.0	39.0
40	44.4	5.8	1.0	38.4

Table S3 Elemental composition (C, H, N, O in wt.%), cellulose and hemicellulose content (wt.%) and molecular weight (M_w, kg mol^{-1}) of extracted fibrous material after NaOH and NaOCl treatment from fermentation residue after 0 to 40 days of anaerobic digestion.

Days of Anaerobic digestion	C [wt.%]	H [wt.%]	N [wt.%]	O [wt.%]	Cellulose [wt.%]	Hemicellulose [wt.%]	M_w [kg mol^{-1}]
0-1	41.58	6.24	0.05	46.55	72.2 ± 2.0	16.3 ± 2.0	312
5	43.01	6.39	<0.05	44.90	69.6 ± 0.1	15.6 ± 1.0	319
10	42.38	6.15	<0.05	46.32	71.5 ± 1.0	8.8 ± 0.9	319
20	43.11	6.16	<0.05	45.03	68.1 ± 0.7	4.5 ± 1.1	302
30	43.67	6.41	0.05	44.30	66.4 ± 0.7	9.0 ± 1.9	371
40	43.46	6.02	0.08	43.91	67.3 ± 2.2	5.2 ± 1.8	341

Table S4 Elemental composition (C, H, N, O in wt.%) of the neutralised and dried residue after alkaline treatment of elephant manure (April (0-1)) and fermentation residue after 5 to 40 days of anaerobic digestion.

Days of Anaerobic digestion	C [wt.%]	H [wt.%]	N [wt.%]	O [wt.%]
0-1	21.72	2.73	0.72	24.47
5	26.76	3.31	1.04	24.97
10	24.80	3.13	0.92	25.14
20	28.61	3.45	1.21	24.97
30	19.51	2.41	1.05	20.37
40	20.50	2.49	1.11	21.33

Table S5 Tensile strength (σ, MPa), elastic modulus (E, GPa), strain to failure (ε, %), fibre diameter (µm, nm) of the produced paper of cellulose extracted from elephant manure of elephant manure collected in April (0-1), June (0-2) and reference material from kraft pulp (KP).

Days of AD	Cycles	σ [MPa]	E [GPa]	ε [%]	Fibre diameter	Energy Consumption [kWh/kg]
0-2	1	56 ± 4	7.5 ±1.3	1.39 ± 0.25	81 ± 29 nm 10 ± 4 µm	0.3 ± 0.1
0-2	2	79 ± 6	10.2 ± 1.0	1.39 ± 0.15	46 ± 9 nm 9 ± 4 µm	0.5 ± 0.1
0-2	5	102 ± 3	9.7 ± 0.8	2.62 ± 0.23	47 ± 12 nm	1.8 ± 0.6
0-2	7	118 ± 3	11.6 ± 0.9	1.78 ± 0.15	35 ±11 nm	2.9 ± 1.0
0-2	10	130 ± 8	13.2 ± 1.7	1.71 ± 0.27	40 ± 16 nm	5.2 ± 2.1
0-3	10	96 ± 5	10.9 ± 1.4	2.84 ± 0.38	44 ± 21 nm	
KP	1	16 ± 2	2.8 ± 0.4	1.39 ± 0.24	19 ± 5 µm	0.1 ± 0.1
KP	2	30 ± 2	5.0 ± 0.9	1.86 ± 0.52	15 ± 5 µm	0.3 ± 0.1
KP	5	57 ± 7	9.1 ± 2.4	2.16 ± 0.54	56 ± 19	1.1 ± 0.3
KP	7	61 ± 2	8.1 ± 0.5	2.95 ± 0.45	47 ± 10	2.5 ± 1.3
KP	10	102 ± 6	11.4 ± 0.4	3.65 ± 0.48	43 ± 13	4.2 ± 2.0

Supplementary Graphs

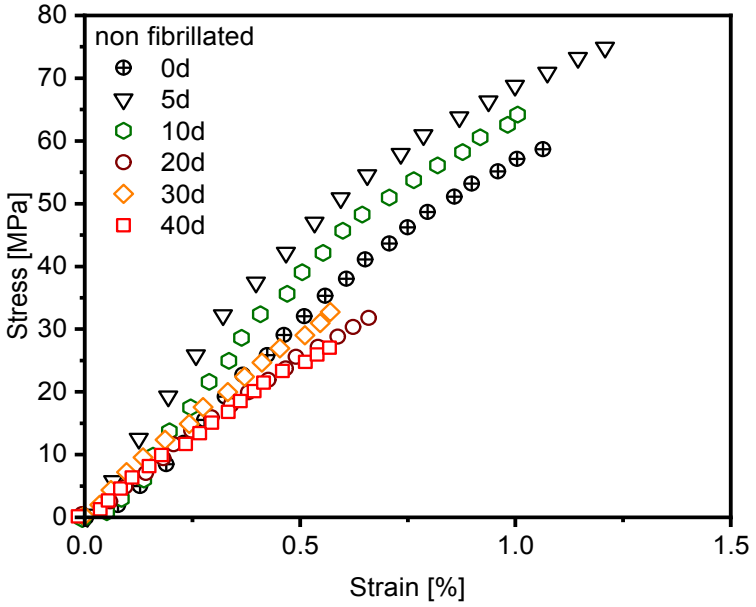

Fig S1 Representative stress-strain curves of papers produced using microfibrillated (non fibrillated) lignocellulosic fibres extracted from elephant manure (crossed dot) and fermentation residue after 5 (black triangle), 10 (green pentagon), 20 (brown circle), 30 (orange diamond) or 40 (red square) days of AD.

Publication II

Horsepower: Horse manure as resource for biogas and nanolignocellulosic fibres

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Abstract

Nanofibrillated cellulose (NFC) is a bio-based material with key applications in composites, water filters and as emulsifiers. Despite extraordinary properties of NFC its affinity to water is still a challenge, as water uptake negatively influences its integrity. To mitigate the impact of water, hydrophobicity is often imparted by chemical modification or by addition of hydrophobic additives. Lignin, a major component of plant biomass, is a natural hydrophobiser. Anaerobic digestion of biomass for the production of biomethane allows to up-concentrate lignin in the fermentation residue still containing lignocellulosic fibres. We show that horse manure used as substrate for biogas production allows for efficient extraction of nanolignocellulose fibres (LCNF) with high lignin content allowing for additional valorisation of the waste material through integrated biogas production. A biogas yield of 207 L_N kg_{VS}^{-1} with a methane concentration of 65% was achieved. The fermentation residue was used as raw material to isolate LCNFs in yields of up to 41% with lignin contents between 23 to 29 wt% depending on fermentation time. Nanopapers produced from LCNFs possessed tensile strength and moduli of 45 to 91 MPa and 7 to 8 GPa, respectively, as compared to 130 MPa and 13 GPa for pure NCF nanopapers. The increased lignin content was responsible for decreased water absorption capacity of the produced papers.

Keywords: Biogas, nanolignocellulose, mechanical properties, up-cycling

1. Introduction

Increasing interest in production of sustainable materials demands for a cheap and abundant raw material. Accordingly, lignocellulosic biomass (LC) was reintroduced as sustainable alternative to petrol based polymers. LC contains cellulose, hemicellulose and lignin as major components, next to pectin and a small fraction of extractives (Hendriks & Zeeman, 2009). In the interest of sustainability in particular agricultural waste streams were investigated as potential raw materials source (Reshmy et al., 2022).

Animal manure is agricultural residue, used as organic fertiliser source. The horse agribusiness is increasing in economic value. The transition of the traditional role of horses for work or transportation shifted to leisure and sport. This and the increasing urbanization has shifted the location of horse stables from the countryside to urban areas (Rantala et al., 2018). This causes environmental challenges related to manure management. Horses produce 18 to 25 kg faeces per day in addition to 8 to 10 kg beddings, depending on feed, bedding and cleaning systems (Böske et al., 2014). Due to strict fertilizer regulations in the EU, only a limited amount of the manure can be used as fertilizer per hectare rendering manure disposal cost intensive (Directive 91/676/EEC, Directive 2016/2284, Directive 2003/35/EC). The majority of horse businesses are small and struggle to make their operations profitable. A manure management system providing the opportunity to valorise horse manure effectively in an environmentally sustainable way would be very beneficial; utilising horse manure for the energy and materials production is one such possibility (Weiland et al., 2021). During anaerobic digestion lignocellulosic biomass is converted into biogas, which can be used for the energy production. The methane yield depends on several parameters, such as substrate type, composition of the microbial consortium, pH or temperature (Kalaiselvan et al., 2022). Cellulose and hemicellulose preferably are degraded prior to lignin due to their better accessibility while lignin degradation takes place mainly under aerobic conditions (Li et al., 2018). This results in up-concentration of lignin in the fermentation residue. The fermentation residue is a sludge (Bauer et al., 2009) still containing lignocellulosic fibres, an unconventional source for the more effortless extraction of nanofibrillated cellulose (NFC) (Krexner et al.,

2022; Weiland et al., 2021). Nanocellulose forms have stayed in the research focus for the past two decades (Candan et al., 2022). In order to obtain NFC, mechano-chemical treatment (pulping) of lignocellulosic biomass is followed by chemical or mechanical defibrillation (Pradhan et al., 2022). During pulping, cellulose is separated from the other plant biopolymers contained in biomass, i.e. lignin is usually removed using potentially harmful chemicals and then incinerated for energy generation causing significant CO_2 emissions (Sun, 2020).

One major obstacle to be overcome for broad applications of NFC for instance in barrier coatings (Österberg et al., 2013) is their susceptibility to water. One possibility to tackle this is by surface modification (Lee et al., 2014; Thomas et al., 2018) or adsorption of hydrophobic polymers (Kontturi et al., 2017). Avoiding removal of lignin, known for its hydrophobicity, during pulping provides the additional advantage of using less chemicals during the extraction process and simplifies the pre-treatment. Moreover, retaining lignin on extracted fibres increases hydrophobicity and adds functionality, such as methoxy-, hydroxyl- or carboxyl groups, broadening the application possibilities of these fibres (Bertella & Luterbacher, 2020).

We investigated horse manure as raw material for the consecutive production of biogas and NCF containing high amounts of lignin. The fermentation residue was extracted using a mild chemical treatment to obtain lignocellulosic fibres, which were mechanically refined to obtain LCNFs. Papers produced from them were characterized with regards to their water interaction and mechanical properties to investigate the influence of anaerobic digestion time on the quality of produced LCNFs.

2. Materials and Methods

We collected fresh horse droppings (30 kg) in July 2021 from Reitclub Tulln (Austria) taking care that as little as possible bedding was included. Neutral detergent solution concentrate, acid detergent solution powder, triethylene glycol and nylon bags were purchased from Ankom Technology (Macedon, USA) and used as received. NaOH (99.6%, Sigma Aldrich), H_2SO_4 (95%, Sigma Aldrich), HCl (95%, Sigma Aldrich), decahydronaphthalene (Merck), peracetic acid (Sigma Aldrich), Ascorbic acid (Nutribiotic Immunity, USA) and sodium sulfite (Sigma

Aldrich) were used as received. Microcrystalline cellulose, used as reference for biogas production, was purchased from Sigma Aldrich. The inoculum for biogas experiments was a blend of two inocula from biogas plants located in Margarethen am Moos and Ziersdorf (Austria).

2.1 Specific methane and degradation yield of horse manure

Dry mass (DM), volatile solid (VS) and ash content of fresh horse manure were calculated using **equations 1** to **3**. DM was determined by drying a quintuple of 3 g (m_{wet}) of horse manure at 105°C for 24 h (m_{dry}). Subsequently the dried material was heated in a muffle oven to 550°C for 5 h ($m_{550°C}$) to determine the ash content of the manure substrate (ash_m).

$$\mathrm{DM\ (wt\%)} = \frac{\mathrm{m_{dry}}}{\mathrm{m_{wet}}} \cdot 100 \quad (1)$$

$$\mathrm{ash_m\ (wt\%)} = \frac{\mathrm{m_{550°C}}}{\mathrm{m_{dry}}} \cdot 100 \quad (2)$$

$$\mathrm{VS\ (wt\%)} = 100 - \mathrm{ash} \quad (3)$$

The biogas production experiments were performed according to standard VDI 4630. 200 g of inoculum (4 wt% dry matter (DM)) and horse manure (3 g wet mass) were mixed in Eudiometer batch fermenters. The substrate and the inoculum mix was held at 37.5°C (mesophilic conditions) for 40 days. The methane and carbon dioxide content were measured using a portable gas analyser (Dräger X-am 7000, Dräger, Lübeck, Germany) and their volumes were normalised to 273.15 K and 101.33 kPa per kilogram of volatile solids ($L_N\ kg_{VS}^{-1}$). Microcrystalline cellulose was used as control substrate.

To investigate the degradation yield of the structural components, 500 g of horse manure was added into nylon bags (Ankom Technology) and immersed in inoculum in a ratio of 2:1. Samples were taken after 5, 10, 20, 30 and 40 days of AD, respectively, and washed thoroughly with deionised water. The samples were dried at 60°C in an oven until constant weight. All experiments were performed sixfold. The yield of residual biomass (degradation yield, $yield_{deg}$) was calculated as follows:

$$yield_{deg}\ (\%) = \frac{m(after\ AD)}{m_{dry}} \cdot 100 \quad (4)$$

2.2 Extraction of lignocellulosic fibres from horse manure and LCNF production

To extract lignocellulosic fibres horse manure and fermentation residue (20 g DM/L) was heated for 30 min at 85 °C in water and the remaining fibrous material washed over a sieve (20 µm mesh size). The obtained fibres were subsequently blended for 3 min (Tristar, BL-4473 VitaPower2000) in 400 mL water, filtered and resuspended in 1 M NaOH and heated to 65 °C for 3 h. The obtained material was washed over a sieve (20 µm mesh size) until neutral pH. The extraction yield is the mass ratio of the dry fibrous material (m (after extraction)) over the dry mass of the material before biogas production (m_{dry}) (**Equation 5**).

$$yield_{extr}\ (\%) = \frac{m(after\ extraction)}{m_{dry}} \cdot 100 \quad (5)$$

LCNF were produced by grinding the extracted fibres (50 g DM as 3.5% suspension) 20 times using a disc mill (Granomat JP 150, Fuchs, Switzerland).

2.3 Extraction of NFC from horse manure

To produce cellulosic fibres from horse manure 3.4 g DM of LCNF after 0 days AD was suspended in 600 mL of peracetic acid (4 wt% at pH 4.8). The suspension was stirred at 50 °C for 48 h. The suspension was then centrifuged for 10 min at 8000 rpm (Hermle Z36 HK, Germany) and the supernatant discharged. The procedure was repeated again, and subsequently ascorbic acid was added. The suspension was centrifuged and resuspended in water until the distinct smell of acetic acid was not detectable anymore. The NFC yield is the ratio of the received dry NFC (m_{NFC}) over the dry mass of the material before biogas production (m_{dry}) (**Equation 6**).

$$yield_{NFC}\ (\%) = \frac{m_{NFC}}{m_{dry}} \cdot 100 \quad (6)$$

2.4 Chemical and physical characterization of fermentation residue and extracted material

Horse manure, fermentation residue and the extracted material (dried at 60 °C for 24 h) were homogenized for 9 min using a cryomill (CryoMill, Retsch, Haan, Germany) at a frequency of 20 Hz. The water content was determined (w_s) by Karl Fischer titration (Mettler Toledo V20,

Columbus, Ohio, USA). The cellulose, hemicellulose and acid detergent lignin contents were determined utilizing the method of Naumann and Bassler.(Naumann & Bassler, 1976; Van Soest & Wine, 1967) In short: 0.5 g of sample was refluxed for 1 h in 50 mL neutral detergent solution (NDF) (30 g neutral detergent powder, 1 mL decahydronaphthalene, 0.25 g sodium sulphite and 5 mL triethylene glycol in 500 mL water) to determine the cellulose, hemicellulose and lignin content. The insoluble residue was filtered using a glass frit (Por. 3), washed with water and dried (105 °C for 12 h). The ratio of the residual material was calculated using **Equation 7**. Analogue to the NDF treatment, 0.5 g of sample was treated with acid detergent fibre solution (ADF) (10 g acid detergent powder and 0.5 mL decahydronaphthalene in 500 mL 0.5 M sulphuric acid) to determine cellulose and lignin content. The dried residue from ADF was subsequently treated with 72% H_2SO_4 to determine the lignin content (ADL) (**Equation 8**). The residue of ADL was heated to 500 °C for 3 h and the residue labelled as ash content of the extracted fibres (ash_f) (**Equation 9**).

$$\mathrm{NDF/ADF\ (wt\%)} = \frac{m_{NDF/ADF}}{m_S - (m_S \cdot w_S)} \cdot 100 \quad (7)$$

$$\mathrm{ADL\ (wt\%)} = \frac{m_{ADL}}{m_S - (m_S \cdot w_S)} \cdot 100 \quad (8)$$

$$ash_f\ \mathrm{(wt\%)} = \frac{m_{ash}}{m_S - (m_S \cdot w_S)} \cdot 100 \quad (9)$$

To calculate the fibre composition the values were calculated using the ash content corrected values for every fibre component.

Cellulose, hemicellulose and lignin contents were calculated based on **Equation 10**, **11 and 12**

$$\mathrm{Cellulose\ (wt\%)} = \frac{(ADF - ADL)}{(100 - ash_f)} \cdot 100 \quad (10)$$

$$\mathrm{Hemicellulose\ (wt\%)} = \frac{(NDF - ADF)}{(100 - ash_f)} \cdot 100 \quad (11)$$

$$\mathrm{Lignin\ (wt\%)} = \frac{ADL}{(100 - ash_f)} \cdot 100 \quad (12)$$

2.5 Physical and surface properties of LCNFs

LCNF films were produced by diluting the LCNF suspension to 2 wt% with water and pouring the suspension (20 mL) into a glass petri dish, followed by drying at 70 °C for 24 h. The dried

films were coated with a gold layer at 60 mA for 60 s (Leica scd 050/EM QSG 100). SEM micrographs (Zeiss Supra 55VP) were taken at an accelerating voltage of 2 kV and working distance of 7.2 mm. The average fibre diameter was determined using ImageJ (1.53e, https://imagej.nih.gov/ij) from at least 100 single values.

LCNF nanopapers with a grammage of 110 ± 20 g m^{-2} were prepared by filtering a suspension of 1.20 g LCNFs (dry mass) in 300 mL of water (Tristar BL-4473 VitaPower 2000) over a sintered glass frit covered with a filter paper (VWR 413, 125 mm in diameter). The wet filter cakes were pressed in between two layers of blotting paper and baking paper at 120 °C for 15 min using 2 kN force. Dog bone shaped specimens (1BA, ÖNORM EN ISO 527-2, 2012) were cut using a manual cutting press (Zwick ZCP 020). Specimens had a parallel width of 2 mm and an overall length of 25 mm. The mass (m) and the thickness (l) of each specimen were determined using a microbalance (Explorer™ Analytical, OHAUS®) and digital micrometer (AnyiMeasuring), respectively. The envelope density was the ratio of the mass (m) over the surface area (A) of the specimen and the thickness (l) **equation 13**.

$$\rho_e(\mathrm{g\,cm^{-3}}) = \frac{m}{A \cdot l} \quad (13)$$

All samples were conditioned in the laboratory at 23 ± 2 °C and RH 40 ± 5% for at least 24 h prior testing. Tensile tests were performed at a testing speed of 1 mm min^{-1} and 10 mm gauge length using a dual column universal testing system (5969, Instron) equipped with a 1 kN load cell. A non-contact video extensometer (Gig ProE iMETRIUM) was used to measure strain. To avoid slipping and perforation, the specimens were clamped using abrasive paper taped on cardboard in the metal jig. The elastic modulus E was analysed in the linear elastic region as a secant between stress values separated by 0.2% strain.

The paper skeletal density (ρ_s) was measured using helium gas displacement pycnometry (Micromeritics AccuPyc II 1340) with a chamber volume of 1 cm^3. The porosity (Φ) was calculated using **Equation 14**.

$$\Phi\,(\%) = \left(1 - \frac{\rho_e}{\rho_s}\right) \cdot 100 \quad (14)$$

Static water contact angles were determined using a drop shape analyser (DSA30, Krüss GmbH, Hamburg, Germany). 5 µL droplets of water were placed on the paper surface and the initial contact angle was determined after dosing. Further contact angles were recorded after 15, 30, 60 and 120 s.

The water absorption capacity of LCNF nanopapers (20 mm x 5 mm) conditioned at 20 °C and 44% RH for 24 h was determined after water immersion for 24 h. The conditioned samples were weighed prior to (m_1) and after removal from water (m_2). Care was taken to remove excess water with a paper cloth. The water adsorption capacity was calculated using **Equation 15.**

$$w_{H2O}(wt\%) = \frac{m_2 - m_1}{m_1} \cdot 100 \quad (15)$$

3. Results and discussion

3.1 Biogas Production

The fresh horse manure had a volatile solid content of 73 wt%, which is lower than those reported in literature. Pure horse dung and horse dung with stable bedding or straw were reported to have a VS content of 83-88 wt% (Böske et al., 2014) and 80-89 wt% (Kusch et al., 2008; Wartell et al., 2012), respectively. Differences are caused by variations of imbedding material but also by seasonal feed availability. The higher the volatile solid content, the higher the fraction of organic compounds that can be converted during anaerobic digestion. The ash content of the unwashed horse manure was 27 wt%, which is higher than reported in literature (3-10 wt%) (Chong et al., 2019; Lundgren & Pettersson, 2009) and was attributed to the presence of sand and stones (Boufi, 2017). The ash content of the washed material was significant lower (7 wt%) but was increasing during anaerobic digestion up to 13 wt% due to degradation of fibrous material and therefore increasing the share of inorganic fraction in the fermentation residue. The “degradation yield” of horse manure prior to AD (0 days) was 83 wt%; this was attributed to materials loss caused by washing of the manure, which was necessary to produce a comparable reference material (Table 1). This indicated that 17% of

the raw material were water soluble compounds. The fibre composition of washed horse manure was 48 wt% for cellulose (Cel), 21 wt% for hemicellulose (HCel) and 29 wt% for lignin (ADL) comparable to values found in the literature (Table 1) (Waksman & McGrath, 1931). After 40 days of AD only 60% of the initial material remained due to conversion of the compounds of horse manure into methane and CO_2. Over the course of 40 days the hemicellulose and cellulose fibre fraction decreased by 37% and 9%, respectively, while the lignin content increased significantly by 50% (42 wt% after 40 days of AD). Especially in the first days, HCel is degraded to a greater extent than Cel, due its higher accessibility for bacterial degradation. Lignin is significantly up-concentrated in the material due to its unflavoured degradation in anaerobic conditions (Li et al., 2018).

Over the first 16 days of AD already the majority of the biogas and methane (185 $L_N\ kg_{VS}^{-1}$and 115 $L_N\ kg_{VS}^{-1}$) were produced. Increasing the time in the fermenter from 16 to 40 days resulted in a cumulative biogas and methane production of 207 and 135 $L_N\ kg_{VS}^{-1}$, respectively. Our data in this study fit well with the yields (164 to 277 $L_N\ kg_{VS}^{-1}$) (Kusch et al., 2008; Mönch-Tegeder et al., 2013). The inconsistent biogas and methane yields found in literature are mainly affected by feeding intensity, composition of forages and bedding material (Böske et al., 2014; Mönch-Tegeder et al., 2013).

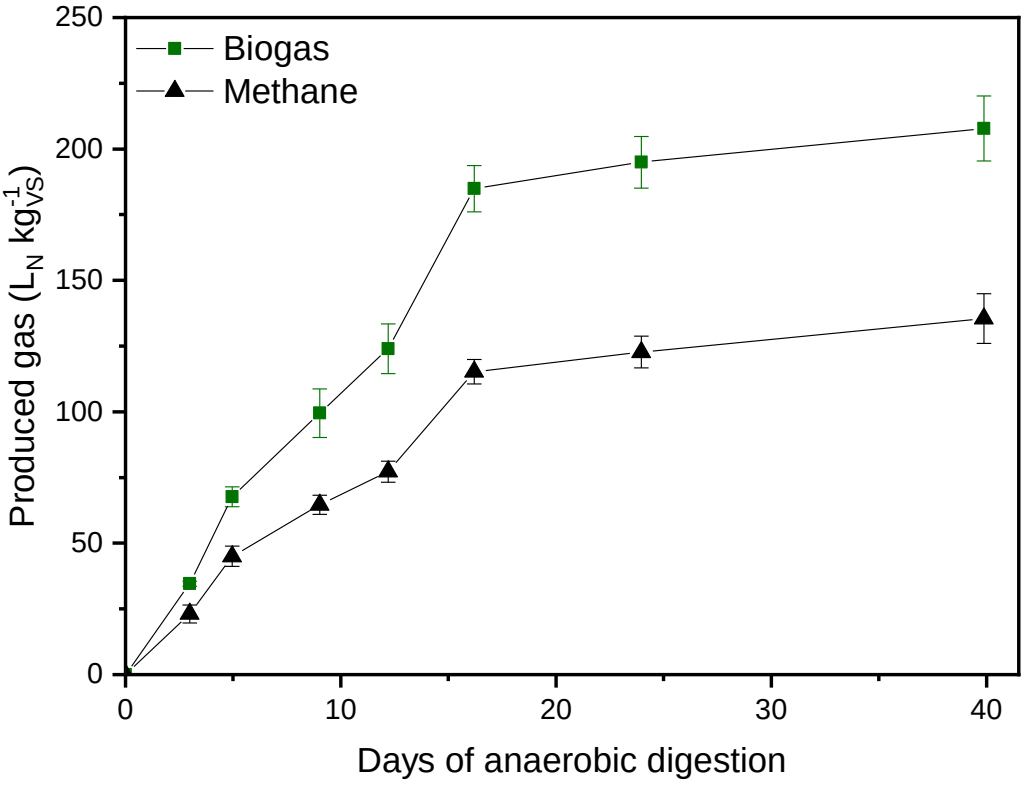

Figure 1 Biogas (green squares) and methane yield (black triangle) over the course of 40 days using horse manure as substrate.

Table 1 Degradation yield, fibre ash content (ash_f) Fibre composition of cellulose (Cel), hemicellulose (HCel) and ADL of washed horse dung and fermentation residue after 5 to 40 days of anaerobic digestion

			Fibre Composition		
AD	Degradation yield	ash_f	Cel	HCel	ADL
[d]	[wt%]	[wt%]	[wt%]		
0	83*	7.0	48.3	20.5	27.9
5	74	5.7	48.6	19.6	28.4
10	68	6.7	48.0	17.4	30.1
20	63	5.5	45.7	16.4	32.3
30	59	11.5	43.7	15.2	40.6
40	60	13.4	44.2	12.9	41.9

*fibre yield of washed manure

3.2 Composition of lignocellulose fibres extracted from horse manure and fermentation residue

Washed horse manure and fermentation residue, respectively, were purified using water and 1M NaOH to obtain lignocellulosic fibres. The extraction yield decreased with the duration of anaerobic digestion from 41 wt% to 28 wt%. This can be attributed to the AD process, during which fibrous material is converted into methane and carbon dioxide. During the degradation the fibrous structure is opened by bacterial action resulting in smaller molecules, which can

then be removed easier by chemical treatment. The ash content decreased significantly compared to the non-extracted material (Table 1) due to removal of sand and stones, but as well as the removal of contained minerals. The alkaline treatment removed hemicellulose (HCel)(Sun et al., 2004) resulting in all samples a constant hemicellulose contents of approx. 10 wt%. The cellulose content decreased by approx. 13% over the course of 40 days AD (Table 2). Cellulose is stable in alkaline solution and was therefore not removed by the chemical treatment but only by anaerobic digestion. Already after 20 days of AD lignin was up-concentrated by 28% (from 23.1 to 28.8 wt%) due to the resistance of lignin to anaerobic bacterial degradation. During this time the majority of the biogas has been produced, hence increasing the residence time in the fermenter further results only in a small increase of the gas yield at the expense of fibre yield (approx. 5% less). Increasing residence time does not significantly affect the lignocellulosic fibre composition (Table 2).

Table 2 Fibre content expressed as extraction yield ($Yield_{extr}$), fibre composition (Cel, HCel, ADL) and fibre diameter of LCNFs produced from horse manure and anaerobically digested horse manure (5 to 40 days).

			Fibre composition			
AD	$Yield_{extr}$	Ash_f	Cel	HCel	ADL	Fibre diameter
[d]	[wt%]	[wt%]	[wt%]			[nm]
0	41	1.6	64.5	11.7	23.1	47 ± 28
5	39	1.1	63.8	12.2	22.7	19 ± 19
10	34	1.0	61.2	9.9	26.2	26 ± 23
20	32	0.5	58.9	10.3	28.8	51 ± 33
30	27	0.9	57.0	10.2	29.0	39 ± 14
40	28	2.1	56.7	11.0	29.1	50 ± 27

3.3 LCNF nanopaper properties

The LCN fibril average diameter (Table 2) after mechanical grinding remained constant within errors around 40 nm. These nanofibers were used to produce nanopapers, which had envelope densities around 1 g cm^{-3} and skeletal densities of approx. 1.41 g cm^{-3} resulting in paper porosities of 20 to 30% (Table 3). The paper densities did not depend on the lignin content. Similar findings for nanopapers made from LCNFs were reported; no dependency of the nanopaper density on the lignin content (Rojo et al., 2015). They proposed a model stating that during hot-pressing lignin acts as matrix covering the cellulose fibrils, independently of

the lignin content. Representative stress-strain curves of LCNFs nanopapers (Figure 2) are characteristic of rather brittle materials. Nanopapers produced from reference LCNF extracted from horse manure (without AD) had a tensile strength of 65 MPa, a modulus of 8 GPa and a strain to failure of 1.2% (Table 3). Nanopapers produced from LCNF extracted from fermentation residue after 5 and 10 days of anaerobic digestion had significantly higher tensile strengths (91 and 88 MPa) likely due to smaller fibre diameters. Nanopapers produced from LCNF after 20 and 30 days of AD exhibited approx. 60 MPa and further decreased to 45 MPa. By increasing lignin content (up to 29 wt%) and further reduction in cellulose content aromatic structures of lignin hindered the formation of hydrogen bonds between the cellulose fibrils resulting in a decrease in tensile strength (Lin et al., 2011). The strains to failure of all nanopapers were rather similar and in line with values reported for cellulose nanopapers.(Hervy et al., 2017) The modulus of the produced nanopapers was constant within error with a trend to lower moduli for papers with higher lignin content. An increasing lignin content, acting as a matrix for cellulose fibrils, results in a lower content of stiff cellulose fibrils within the nanopapers affecting the measured modulus. Nanopapers, which had a tensile strength, a modulus and a strain to failure of 130 MPa, 12.6 GPa and 1.7%, respectively could be produced from bleached material (0b). The removal of lignin to a lignin content of 0.7 wt% enables the formation of a higher number of hydrogen bonds leading to a strong fibre network. The hemicellulose and cellulose content were 6.8 wt% and 92.1 wt%, respectively. By introducing a bleaching treatment the extraction yield 49% (21 wt%) lower compared to the extraction yield of LCNFs extracted from horse manure without anaerobic digestion.

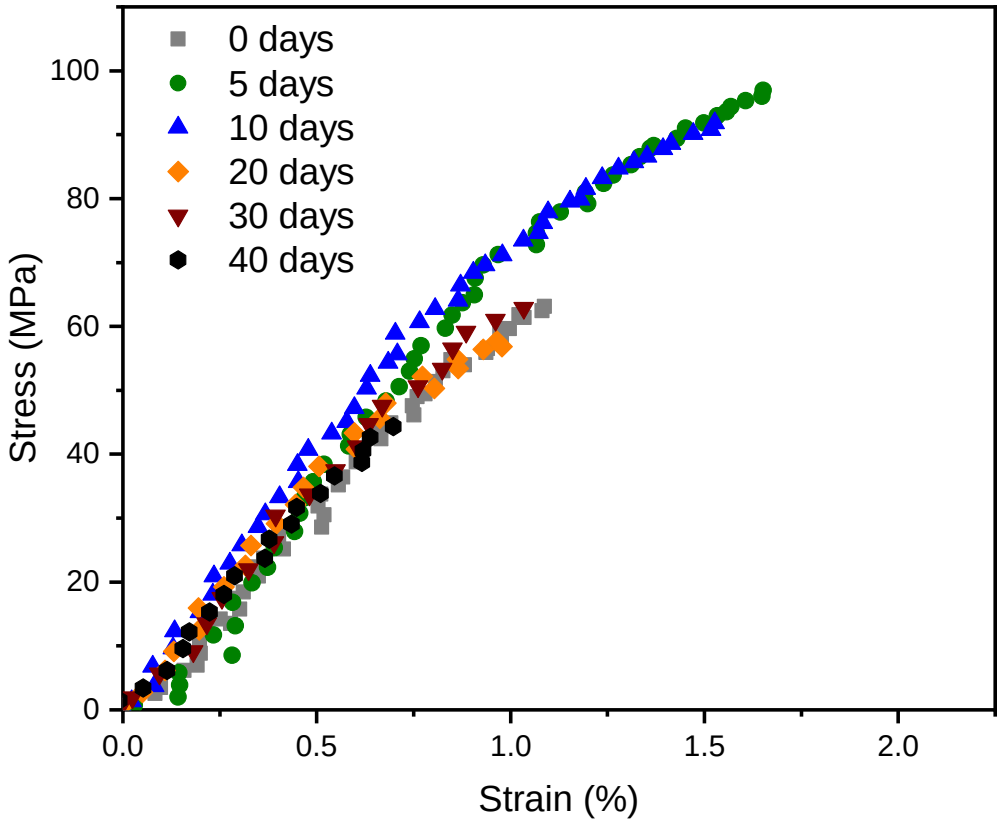

Figure 2 Representative stress-strain curves of nanopapers produced from LCNF extracted from horse manure (gray square) and fermentation residue of 5 (green circle), 10 (blue triangle up), 20 (orange diamond), 30 (red triangle down) and 40 days (black pentagon) of AD.

Our LCNF papers exhibited comparable mechanical properties to the few other LCNF papers found in the literature albeit produced from different raw materials. LCNF papers produced from fibrils extracted from Scots pine chips with lignin contents of 20 to 24 wt% had tensile strengths of 71 to 125 MPa (Hanhikoski et al., 2020), while papers produced from LCNF extracted from bark with 23 wt% of lignin had a similar tensile strength of 90 MPa and a modulus of 7 GPa but a slightly higher strain to failure of 3% (Huang et al., 2019). Our nanopapers produced from LCNF extracted from fermentation residue after 5 and 10 days of AD had a very similar property profile, but we used an unconventional non-wood fibre source collected by horses and with the added advantage that our process also yielded biogas. Using animals as collectors and pre-treater (grinding and digestion) for lignocellulosic biomass and the subsequent anaerobic fermentation for biogas production allows for significant reductions in environmental impact categories associated with the production of common nanocellulose (Krexner et al., 2022; Weiland et al., 2021).

Table 3 Mechanical properties namely tensile strength σ, modulus E and strain to failure ε, as well as physical properties, envelope ρ_e, skeletal density ρ_s and porosity Φ of nanopapers produced from LCNF extracted from horse manure and fermentation residue after various durations of AD.

	Mechanical properties			Density		Porosity
AD	σ	E	ε	ρ_e	ρ_s	Φ
[d]	[MPa]	[GPa]	[%]	[g cm^{-3}]	[g cm^{-3}]	[%]
0	65 ± 16	8.0 ± 0.7	1.2 ± 0.3	1.01 ± 0.03	1.42 ± 0.003	29
5	91 ± 9	7.7 ± 1.0	1.6 ± 0.3	1.08 ± 0.03	1.43 ± 0.009	24
10	88 ± 6	7.1 ± 1.0	1.9 ± 0.2	1.07 ± 0.03	1.42 ± 0.003	25
20	58 ± 11	7.9 ± 0.7	1.0 ± 0.1	1.06 ± 0.13	1.42 ± 0.004	26
30	61 ± 11	7.0 ± 1.3	1.2 ± 0.3	1.10 ± 0.03	1.39 ± 0.002	21
40	45 ± 11	7.0 ± 0.6	0.9 ± 0.3	1.01 ± 0.08	1.43 ± 0.004	29
0b	130 ± 9	12.6 ± 0.7	1.7 ± 0.7	1.19 ± 0.03	1.43 ± 0.001	17

To investigate the interaction with water, first contact angle measurements were performed (Table 4). The initial contact angle measured directly after deposition of the water droplets ranged between 63° and 75° for all LCNF nanopapers. With increasing resting time of the water droplet (up to 120 s) the contact angle only slightly decreased and the water droplets did not completely imbibe into the LCNF nanopapers – in contrast to a pure NFC or bacterial cellulose nanopapers with initial water contact angles of about 20 to 40° into which water is absorbed completely (Lee et al., 2011; Österberg et al., 2013; Vuoti et al., 2013; Wang et al., 2022). Also the nanopapers produced from bleached cellulosic fibres had a not significant lower contact angle than those of LCNF (59° to 54°). Comparable LCNF papers produced with lignin contents of 4 wt% and 14 wt% exhibited similar static water contact angles (61° and 78°) (Rojo et al., 2015). The increased lignin content therefore significantly influenced the hydrophobicity of cellulose fibrils, also indicating that lignin acts as matrix for the cellulose fibrils.(Ferrer et al., 2012) The water uptake (w_{H2O}) within 24 h was tested (Table 4) showing a significant reduction by 30% with increasing lignin content, which is also responsible for the reduced wettability of the nanopapers. None of the produced LCNF papers lost their integrity or disintegrated within 24 h immersion into water. Nanopapers produced from bleached nanofibers extracted from horse manure had a 34% higher water uptake than LCNF papers produced from extracted material from horse manure without anaerobic digestion.

Table 4 Static water contact angles (Θ_w) for LCNF papers produced fibrils extracted from horse manure and fermentation residue of 5 to 40 days AD. Drop residence times of 0 to 120 s were recorded. Water absorption capacity (w_{H_2O}) determined after 24 h immersion into water.

	Θ_w					
AD	0	15	30	60	120	w_{H_2O}
[d]			[°]			[wt%]
0	71 ± 7	69 ± 6	68 ± 7	68 ± 5	65 ± 5	125 ± 21
5	65 ± 6	63 ± 6	62 ± 6	61 ± 7	59 ± 7	112 ± 11
10	67 ± 4	66 ± 5	65 ± 5	64 ± 5	61 ± 5	110 ± 6
20	63 ± 3	61 ± 4	60 ± 4	59 ± 4	56 ± 4	93 ± 6
30	66 ± 4	65 ± 4	64 ± 4	63 ± 4	61 ± 4	84 ± 3
40	75 ± 5	73 ± 5	72 ± 5	71 ± 5	68 ± 6	87 ± 8
0b	59 ± 4	58 ± 4	57 ± 4	56 ± 4	54 ± 4	167 ± 5

4. Conclusion

Horse manure was used as substrate for the production of biogas and the residual fibrous material after anaerobic digestion used to extract nanolignocellulosic fibrils with high lignin contents. Biogas and methane yields obtained were 207 and 135 $L_N\ kg_{VS}^{-1}$, respectively over the course of 40 days, with the majority of biogas being produced in the first 16 days. LCNF yields were 30 to 40% with lignin contents of 29 wt%, demonstrating that it is possible using anaerobic digestion to up-concentrate lignin on cellulosic (nano)fibres. LCNF nanopapers with attractive mechanical properties could be produced. The wettability and water absorption capacity of lignin containing nanopapers was significantly reduced as compared to NFC papers. We demonstrated that anaerobic digestion of horse manure is a suitable treatment to produce value added products besides biogas. By tailoring the residence time in the fermenter it is possible to produce nanolignocellulosic materials with a tuneable cellulose/lignin ratio. Utilizing horse manure in a multistage upcycling process provides the possibility to mitigate the environmental impact of surplus manure by simultaneous valorisation of a waste material.

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Grow it yourself composites: delignification and hybridisation of lignocellulosic material using animals and fungi

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The use of chemical and energy intensive delignification processes in industrial pulping generates cellulosic fibres that are hydrophilic, hygroscopic and functionally restricted to paper and cardboard applications. Here, we propose a bio-based alternative to chemical pulping utilising herbivores to harvest and grind lignocellulosic materials followed by natural fungal growth to delignify and hybridise them to generate hierarchical composite papers with altered, water-repelling surface properties. These papers comprise cellulose and fungal biopolymers produced by cultivation of *T. versicolor* and *P. ostreatus* on elephant manure. Papers with considerably more hydrophobic surfaces were obtained at glucosamine contents as low as 0.1 wt%. Paper tensile strengths and elastic moduli were improved with longer fungal growth periods, spanning several weeks, resulting in comprehensive interfacing of cellulose microfibrils through tough nanoscale fungal chitin-β-glucan networks within the papers. Papers produced from lignocellulosic material colonised with *P. ostreatus* for 16 weeks exhibited the highest tensile strengths and more hydrophobic surfaces than *T. versicolor*. Hybridisation of lignocellulose with fungal biopolymers resulting in improved surface and mechanical properties highlights the extended opportunities of fungal delignification bioprocessing and demonstrates the considerable potential of the fungal biorefinery as an economical, and as of yet underexploited technology.

Introduction

Plant-based lignocellulosic feedstock is generally converted into useful materials, such as paper, cardboard and chemicals, by means of separation and delignification of the constituent fibres in pulp mills or biorefineries. Delignification is designed to remove lignin – a dark brown, amorphous and sticky, non-linear polyphenol – while retaining the carbohydrate polymers, such as cellulose and hemicellulose in the fibre matrix.[1] Pulping is a well-established and widely used industrial process but it is also energy and chemical intensive and results in the generation of undesirable wastes and by-products. Recent studies subsequently focus on alternative utilisations to convert lignin into functional materials[2,3] and avoid CO_2 emissions that emerge from the incineration of extracted lignin to fuel pulp mills.[4] Pulp fibres produced using this method are useful for traditional paper-based products but are not without inherent weaknesses. The most notable deficiency of pulp fibres is their susceptibility to water, which must often be circumvented during product design.[5] The application of a thick polyethylene film on both sides of a milk carton is a classic example of one such method to overcome this problem.[6]

In this study, we introduce an alternative, entirely bio-based approach to industrial pulping resulting in materials with properties that surpass those of present paper products (Fig. 1). First, we make use of animal manure, outsourcing the collection of plant biomass to herbivores that then disintegrate this material into fibres in their digestive system. Elephant manure was used as a model system, due to the elephant's high food intake (up to 150 kg day^{-1}) and their inefficient monogastric digestion system. Feeding mainly on hay, shrubs and grass, their manure has one of the highest contents of lignocellulosic material (approx. 75 wt%) among herbivore manure, which guarantees a high yield of largely intact lignocellulosic fibres whose morphology has nevertheless been altered in the nanoscale for a more favourable eventual isolation of nanocellulose than is possible from virgin fibres.[7,8] Second, lignin is reduced

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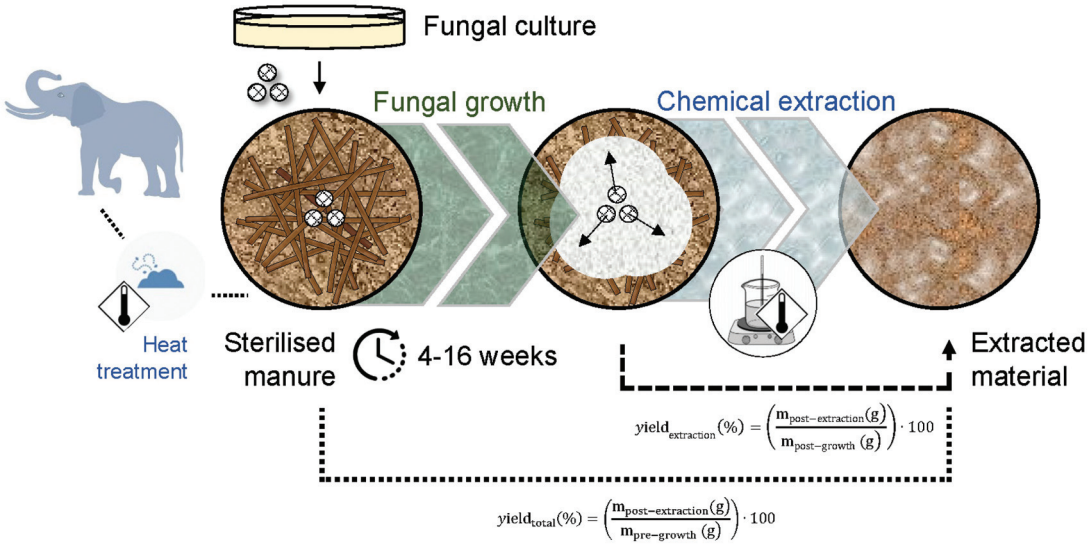

Fig. 1 Biological and chemical treatment of lignocellulosic material derived from elephant manure for paper production. Sterilised manure was inoculated with three 8 mm discs cut from *P. ostreatus* or *T. versicolor* solid tissue cultures for delignification and hybridisation with fungal chitin-β-glucan. After a growth time of 4–16 weeks, the material was deproteinised with NaOH. Extracted material was further processed into papers.

in the excreted fibres using fungal delignification through colonisation with white rot fungi.[9,10] In addition to delignification, the fungal biorefinery also generates a nanoscale network of chitin and β-glucans within the lignocellulosic fibres, altering the interfacial properties in the fibre network for better bonding and stronger papers with water-repellent, hydrophobic surfaces that can be further modified through variations in fungal growth time.

We stress that our approach is distinct from so-called biopulping processes – where fungi or enzymes are applied to achieve partial delignification as a pre-treatment for chemical processing – because here, the chitinous material generated by the fungi is utilised as an integral part of the resulting composite and not simply washed away. Our method is in line with recent trends utilising the potential of fungal growth to produce materials, such as mycelium-derived composites for packaging,[11,12] thermal and acoustic insulation,[13–16] and fungal-derived nanomaterials for use as wound-dressings,[17,18] membranes,[19,20] and other paper-[21–24] and leather-like materials.[25,26] Much of the peculiar properties of these fungi-derived materials are based on the arrangement of semi-crystalline chitin linked with amorphous β-glucan in the fungal cell walls. The composite chitin nanofibre networks within the partially delignified lignocellulosic fibres result in paper with unforeseen properties: it is both strong and tough[24] with hydrophobic surfaces exhibiting water contact angles exceeding 100°.[21,27,28]

The concept shown in Fig. 1 could potentially be viable if adopted by industrial herbivore feeding operations, which produce a considerable amount of surplus manure, parts of which could be used as substrate for cultivation of fungi. Fungal fruiting bodies could be harvested and the remaining substrate including mycelial growth used as feedstock for the production of mycelium composites. The proposed production system also meets UN Sustainable Development Criteria and green chemistry principles through the reduction and prevention of waste by using manure as renewable feedstock. Use of animals as a harvester and preliminary bioreactor for comminution and pre-treatment of low-grade biomass, on which animals feed, would considerably reduce the energy and chemical resources required for the processing of lignocellulosic material compared to conventional paper production processes.

Results and discussion

Chemical and elemental composition of colonised elephant manure

Elephant manure samples colonised with either *P. ostreatus* or *T. versicolor* fungal growth for 8–16 weeks were associated with acid-insoluble lignin (AIL) content reductions of up to about one-third (~7 wt%), although *T. versicolor* growth facilitated greater delignification (a reduction from 21.1 wt% to 14.5 wt% and 23.2 wt% to 16.4 wt% over 16 weeks achieved with *P. ostreatus*) (Table 1). Lignin has been reported to depolymerise into low molecular weight phenolic and aldehyde moieties at temperatures of 160 °C in wet conditions and recondense at 150 °C in dry conditions.[29] The sterilisation process can, however, release cellulosic fibrils from the larger fibres by cleavage of hemicellulose resulting in an increase of smaller molecules available for digestion by the fungus.[30] Enzymes excreted by fungi, such as lignin peroxidase, manganese peroxidase and laccase promote the degradation of lignin in an oxidative process. During the first weeks of colonisation, only minor ligninolytic extracellular action was expected. With increasing

Table 1 Composition (acid insoluble lignin (AIL), extractives and ash content) and yield for extracted elephant manure colonised for 4–16 weeks with *P. ostreatus* and *T. versicolor*. Reference values for elephant manure-derived lignocellulosic material are also included

Fungal species	Growth time (weeks)	AIL (wt%)	Extractives (wt%)	Ash (wt%)	Yield (wt%)
Reference	0	20.7 ± 0.3	0.23 ± 0.11	0.06 ± 0.02	44.8
P. ostreatus	4	23.2 ± 0.2	0.31 ± 0.11	0.05 ± 0.02	60.6
	8	19.7 ± 0.6	0.48 ± 0.07	0.05 ± 0.02	38.1
	12	16.3 ± 0.5	0.54 ± 0.30	0.76 ± 0.06	36.3
	16	16.4 ± 0.3	0.79 ± 0.21	0.79 ± 0.04	17.6
T. versicolor	4	21.1 ± 0.4	0.26 ± 0.13	0.08 ± 0.03	47.0
	8	15.8 ± 0.2	0.53 ± 0.17	1.28 ± 0.15	42.6
	12	19.6 ± 0.3	0.44 ± 0.08	0.09 ± 0.02	36.4
	16	14.5 ± 0.5	1.17 ± 0.33	0.60 ± 0.24	16.5

growth time and expanding hyphal networks the degradation of lignin was accelerated.[31,32] The content of extractives increased with greater fungal colonisation for both species by factors >3 and 5 for *P. ostreatus* (0.23 wt% to 0.79 wt%) and *T. versicolor* (0.23 wt% to 1.17 wt%), respectively, over 16 weeks compared to the uncolonised reference material. Ash content also increased marginally for both species due to conversion of organic matter, as seen in the accumulation of inorganic minerals in the material during fungal growth.[21] The decreasing yield with increasing growth time can be attributed to the degradation of the lignocellulosic material by the fungi, generating CO_2 and H_2O during growth. At the same time, more alkaline soluble compounds were generated, leading to a lower extraction yield, but higher purification.

Carbohydrate analysis indicated changes in glucose content (from 77 to 85 wt% of total carbohydrates for all samples) with increasing fungal colonisation time despite associated ligninolytic and cellulosic enzyme activity, which also degraded hemicellulose and cellulose (Table 2).[31] This could be caused by the presence of β-glucan with increasing fungal biomass, consisting of glucose monomers, which offset the reduction in glucose associated with the loss of cellulose.[33] Within 16 weeks growth time the xylose content, a monomer associated with hemicellulose, decreased by ~34 wt% and ~45 wt% for *P. ostreatus* and *T. versicolor*, respectively. It is noteworthy that hemicellulose is partially removed by alkaline treatment, whereas glucans are known to be alkali resistant.[22] Alkali treatment can also cause chitin to be deacetylated to form chitosan. This can be minimised using low concentrations of NaOH.[19] The increasing fungal biomass content of colonised substrates was indicated by increased N content (0.1–0.3 wt%) in these samples and glucosamine contents that approximately doubled every 4 weeks for the first 12 weeks in both species before tripling in the final 4 weeks of growth. Despite the very high β-glucan content of its hyphae (~86%),[21] *T. versicolor* samples exhibited a higher N (0.2–0.3 wt%) and glucosamine (0.1–1.6 wt%) content compared to *P. ostreatus* (N 0.1–0.2 wt%, and glucosamine 0.1–1.12 wt%) over their growth period. This was most likely attributable to the considerably higher growth rate of *T. versicolor*, resulting in the generation of much higher quantities of fungal biomass over the same period.[34]

Physical and mechanical properties of composite papers

Composite papers comprising lignocellulosic fibres extracted from elephant manure and chitin-β-glucan complex produced by the fungi had higher envelope densities (0.57–0.77 g cm^{-3}) than reference papers produced from pure lignocellulosic reference material (0.57 g cm^{-3}) (Table 3). These envelope densities were lower than those associated with standard copy paper (1.20 g cm^{-3}) but higher than crustacean chitin papers derived from *C. pagurus* (0.46 g cm^{-3}), with values resembling chitin-β-glucan papers produced from fungal (nano)fibrils extracted from *A. bisporus* (0.60 g cm^{-3}) and *D. confragosa* (0.69 g cm^{-3}) fruiting bodies.[23] The skeletal density of lignocellulosic material colonised with *P. ostreatus* and *T. versicolor* was not influenced by the growth time and ranged from 1.42

Table 2 Carbohydrate composition (arabinose (Ara), rhamnose (Rha), galactose (Gal), glucose (Glu), xylose (Xyl), mannose (Man) and glucosamine (GluA)) and elemental composition (C, H, N and O) of extracted elephant manure-derived lignocellulosic materials colonised with *P. ostreatus* and *T. versicolor* fungal growth over 4–16 weeks. Reference values for elephant manure-derived lignocellulosic material are also provided

		Carbohydrate composition (wt% of total carbohydrates)							Elemental composition (wt%)			
Fungal species	Growth time (weeks)	Ara	Rha	Gal	Glu	Xyl	Man	GluA	C	H	N	O
Reference	0	1.9	0.1	1.1	77.2	17.5	2.0	0.1	44.1	6.2	0.2	46.6
P. ostreatus	4	2.0	0.2	1.1	76.9	16.9	2.7	0.1	45.1	6.1	0.3	45.0
	8	1.7	0.1	0.9	78.7	15.2	3.2	0.2	45.3	6.1	0.1	44.6
	12	1.8	0.2	0.9	77.6	16.4	2.7	0.4	45.4	6.2	0.2	43.7
	16	1.0	0.1	0.4	83.3	11.5	2.5	1.2	43.2	6.1	0.2	47.4
T. versicolor	4	1.9	0.2	1.0	75.8	18.3	2.6	0.1	44.6	5.9	0.2	45.2
	8	1.6	0.2	0.9	79.0	15.0	3.0	0.3	44.8	6.2	0.2	43.9
	12	1.6	0.2	0.8	79.7	15.0	2.4	0.5	44.8	6.2	0.2	43.8
	16	0.9	0.0	0.5	84.5	9.7	2.7	1.6	43.1	6.2	0.3	46.6

Table 3 Envelope (ρ_e) and skeletal (ρ_s) density, porosity (Φ), tensile strength (σ), elastic modulus (E), and strain to failure (ε) of composite papers produced using *P. ostreatus* and *T. versicolor* filamentous fungal growth on elephant manure over 4–16 weeks. Reference values for lignocellulosic material extracted from elephant manure are also provided

Fungal species	Growth (weeks)	ρ_e (g cm^{-3})	ρ_s (g cm^{-3})	Φ (%)	σ (MPa)	E (GPa)	ε (%)
Reference	0	0.57 ± 0.01	1.47 ± 0.01	61.2	15.5 ± 0.8	3.6 ± 0.3	0.55 ± 0.05
P. ostreatus	4	0.62 ± 0.03	1.42 ± 0.01	56.5	12.7 ± 1.8	3.7 ± 0.5	0.44 ± 0.05
	8	0.57 ± 0.02	1.44 ± 0.01	60.5	14.6 ± 0.7	3.8 ± 0.2	0.50 ± 0.02
	12	0.59 ± 0.02	1.43 ± 0.01	58.6	13.8 ± 2.3	3.7 ± 0.5	0.46 ± 0.06
	16	0.77 ± 0.02	1.46 ± 0.01	46.9	36.6 ± 3.7	8.0 ± 0.6	0.71 ± 0.12
T. versicolor	4	0.57 ± 0.02	1.45 ± 0.01	60.7	13.3 ± 2.2	3.5 ± 0.5	0.49 ± 0.11
	8	0.62 ± 0.03	1.44 ± 0.01	57.0	16.2 ± 3.0	4.3 ± 0.3	0.47 ± 0.08
	12	0.62 ± 0.03	1.45 ± 0.01	57.3	15.3 ± 1.6	4.4 ± 0.4	0.43 ± 0.03
	16	0.69 ± 0.02	1.45 ± 0.01	52.3	26.4 ± 4.9	5.8 ± 0.4	0.57 ± 0.09

to 1.46 g cm^{-3} and 1.44 to 1.45 g cm^{-3}, respectively (Table 3). Some exceptions existed for *P. ostreatus* composite papers, however, these isolated examples can be attributed to inherent biological variation in fungal growth and raw manure composition. Increases in envelope density were accompanied by ~14% and ~9% reductions in porosity, respectively, for *P. ostreatus* (from 61.2% to 46.9%) and *T. versicolor* (from 61.2% to 52.3%) composite papers over 16 weeks. These reductions in porosity made the composite papers less porous than *C. pagurus* crustacean-derived chitin (67.3%), *A. bisporus* (59.0%) and *D. confragosa* fruiting body-derived chitin-β-glucan (55.0%) papers[23] but much more porous than nanocellulose papers (~20–30%).[35,36]

SEM investigations further revealed the role of fungal activity in hybridisation of composite papers, with mycelium-derived nanoscale chitin-β-glucan networks filling the pores between cellulose microfibrils and interfacing them (Fig. 2). The presence of chitin-β-glucan complexes was noted in all samples after fungal colonisation; however, a homogenous hybridised surface was only observed in composites with a growth time of 16 weeks. The partial delignification as well as the reinforcing effect of the nanofibrous chitin-β-glucan network derived from the fungal mycelium resulted in considerably improved tensile properties of papers produced from lignocellulosic material colonised with fungi for 16 weeks compared with lignocellulosic fibres extracted from manure (reference) (Fig. 3). Papers produced from *P. ostreatus* colonised samples with a growth time of 16 weeks had the highest tensile strengths (37 MPa), ~40% higher than that of papers produced from *T. versicolor* samples with the same growth period (26 MPa) and approximately double that of the reference paper (16 MPa). These improvements were mirrored in the elastic moduli of the materials; *P. ostreatus* composite papers had a modulus of 8.0 GPa after 16 weeks growth, which was again ~40% higher than that of *T. versicolor* samples after the same growth period (5.8 GPa) while the reference paper had a modulus of only 3.6 GPa. Papers derived from lignocellulosic material after 16 weeks of *P. ostreatus* colonisation had a 25–30% higher strain to failure (0.71%) than *T. versicolor* samples with the same growth period (0.57%) and the reference papers (0.55%).

The better tensile properties exhibited by *P. ostreatus* papers over *T. versicolor* composite papers can be partially attributed to their lower porosity. The relationship between improved mechanical properties and the hybridisation effect of the chitin-β-glucan network bridging the lignocellulose microfibres *via* hydrogen bonding was also apparent in the importance of growth time with 4-, 8- and 12-week growth times providing an insignificant reinforcing effect, despite the presence of chitin-β-glucan complex in these papers, followed by considerable strengthening once sufficient interfacing was achieved after 16 weeks (Fig. 2f).

The tensile strengths of papers produced from fibre material extracted from manure colonised for 16 weeks (26–37 MPa) are best described in the context of other lignocellulosic and fungi-derived composite materials that they outperformed, including copy paper (15–40 MPa),[37,38] chemically bleached elephant manure[39] and chemically unmodified hot-pressed mycelium composites comprising cotton or rapeseed straw and *P. ostreatus* or *T. multicolor* fungal growth (0.13–0.24 MPa).[40] They also had higher tensile strengths than pure mycelium-derived nanopapers (1–25 MPa) from *A. arbuscular*, *M. genevensis* and *T. versicolor*.[21] As a point of reference fungal biomass is also only typically associated with tensile strengths of up to 24 MPa,[41–43] illustrating the synergistic effect achieved through interfacing of cellulose microfibres with a nanoscale chitin-β-glucan network. Tensile properties comparable with chitinous nanopapers produced from *A. bisporus* white button mushrooms (97–204 MPa)[13,24] and crustacean shells (70–157 MPa)[24,44,45] or even pure nanocellulose papers (80–140 MPa),[35,46,47] may be achievable through additional mechanical defibrillation of the fibre material to fully exploit the potential of nanometre sized fibres.

Surface properties of composite papers

Composite papers comprising elephant manure-derived lignocellulosic fibres and a fungal mycelium-derived chitin-β-glucan complex exhibited much higher static water contact angles θ (41–85°) than papers produced from lignocellulosic fibres extracted from raw elephant manure, on which water droplets spread and imbibe completely with no measurable contact angle (Table 4). Notably, these considerably more hydrophobic surfaces were already achieved with as little as 4 weeks fungal growth and a glucosamine content of just 0.1 wt%. The extractive content of *P. ostreatus* (0.23 wt%) and *T. versicolor*

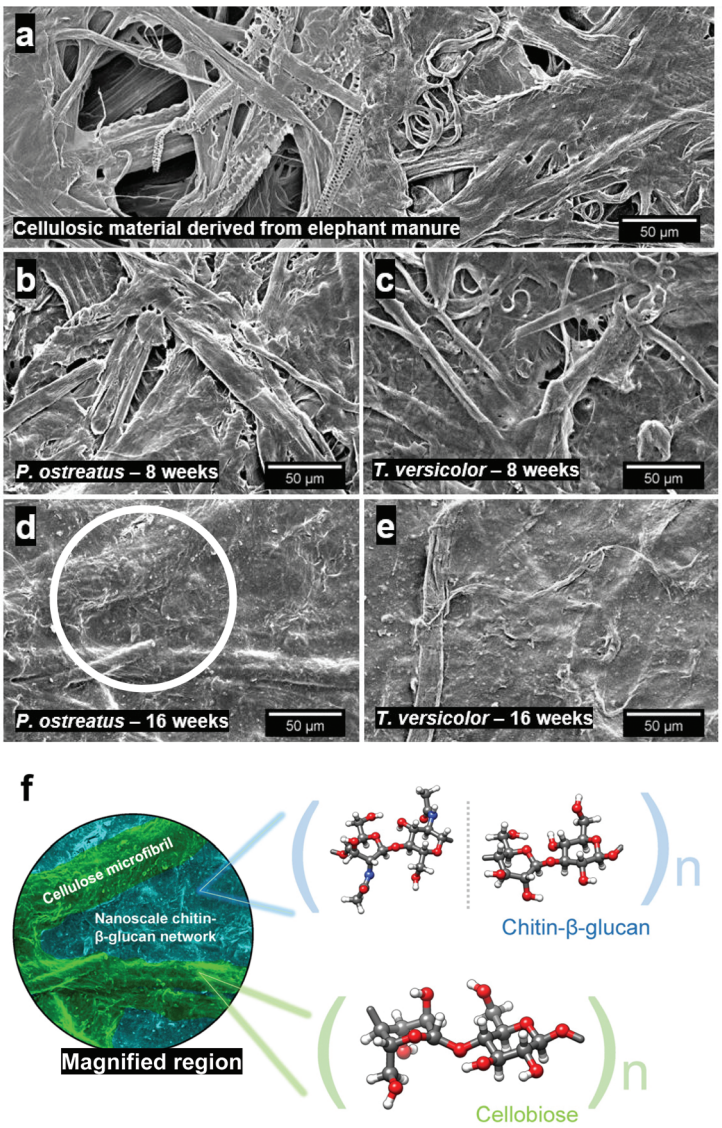

Fig. 2 Characteristic SEM micrographs (500× magnification) illustrating the surface topography of hot-pressed papers produced from (a) lignocellulosic fibres extracted from elephant manure (reference), and fibre material extracted from *P. ostreatus* (b and d) and *T. versicolor* (c and e) colonised manure after growth times of 8 (b and c) and 16 weeks (d and e). Magnified region: The cellulose microfibrils of lignocellulosic material are coloured in green and the nanoscale chitin-β-glucan network bridging the microfibres in blue. Molecular structures of the polymers are also provided (f).

(0.31 wt%) composite papers was also similar to the reference (0.26 wt%) (Table 2). Use of *P. ostreatus* fungal growth to hybridise the lignocellulosic fibers resulted in similar water contact angles to *T. versicolor* samples for most growth periods. At 60 s exposure time the contact angle increased almost linearly with growth time for papers produced from fibres extracted from manure colonised with *T. versicolor* samples, increasing by up to 30° over 12 weeks of fungal growth to a maximum of 74° (Fig. 4). ANOVA indicated significant differences for water contact angles associated with 4- and 8-week growth times compared to each other and 12- and 16-week growth times, however, 12- and 16-week growth times were not significantly different to each other. *P. ostreatus* water contact angles did not vary considerably across the 16-week growth period, retaining a value of 70–80°, with the exception of paper produced from material extracted after 8 weeks of fungal growth, which exhibited lower θ (40–80°). This is likely attributable to inherent inconsistencies in the fungal biomass at the surface, not uncommon due to the biological nature of this growth.[8]

Droplet stability varied across the composite papers but improved with growth time for both fungal species. For *T. versicolor* papers utilising 4- and 8-week growth periods, the contact angle reduced by >30° over the interval between 5 and 60 s due to water imbibition in the paper substrate. For papers produced from fibres extracted after a growth period of 12 and 16 weeks the contact angle dropped by 7° and 2°, respectively.

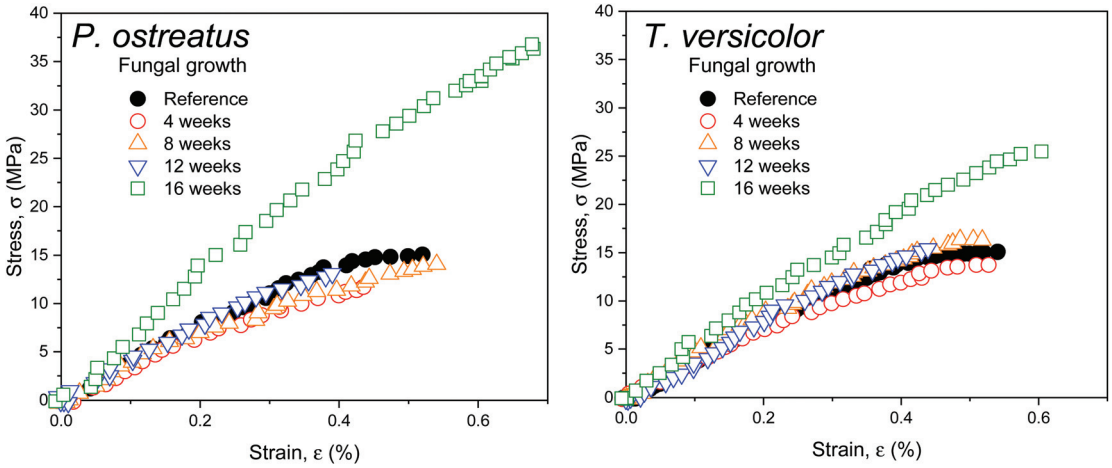

Fig. 3 Representative tensile stress–strain curves for composite papers produced using lignocellulosic fibres extracted from *P. ostreatus* and *T. versicolor* filamentous fungal growth on elephant manure over 4 (red circles), 8 (orange triangles), 12 (blue inverted triangles) and 16 (green squares) weeks. Reference values for papers produced from elephant manure-derived lignocellulosic fibres are also provided (black solid circles).

Table 4 Static water contact angles (θ_{water}) over 5, 30 and 60 s of composite papers produced from lignocellulosic fibres extracted from *P. ostreatus* and *T. versicolor* filamentous fungal growth on elephant manure over 4–16 weeks. Reference values for elephant manure-derived lignocellulosic material are also provided

		Water contact angle, θ_{water} (°)		
Fungal species	Growth (weeks)	5 s	30 s	60 s
Reference	0	0	0	0
P. ostreatus	4	81 ± 7	75 ± 7	68 ± 9
	8	76 ± 7	53 ± 13	41 ± 12
	12	80 ± 8	72 ± 13	63 ± 18
	16	77 ± 6	74 ± 7	72 ± 7
T. versicolor	4	77 ± 7	61 ± 11	44 ± 11
	8	85 ± 7	66 ± 12	55 ± 14
	12	83 ± 9	81 ± 9	76 ± 13
	16	76 ± 10	75 ± 9	74 ± 8

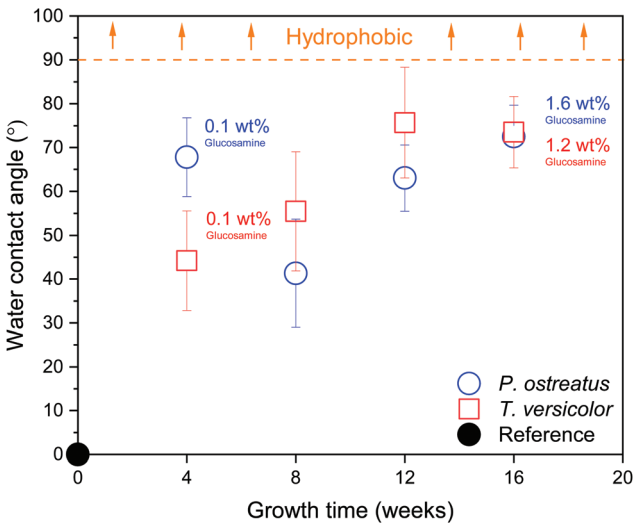

Fig. 4 Static water contact angle (°) measured after 60 s on composite papers produced from fibres extracted from manure as a function of growth time (4, 8, 12 and 16 weeks) for *P. ostreatus* (blue circles) and *T. versicolor* (red squares) and hot-pressed papers (reference).

ANOVA indicated significant differences for exposure time dependent contact angles of 4- and 8-week growth periods but no significant difference for a 16-week growth time. Water droplets on papers derived from lignocellulosic material colonised with *P. ostreatus* fungal growth generally exhibited lower stability than those on *T. versicolor* papers, with a reduction of 35° for a growth time of 8 weeks (by 30° over 60 s for *T. versicolor*) and 17° and 5° reductions over 60 s for 12- and 16-week growth times, respectively. ANOVA indicated more significant differences in the mean contact angles of 5 to 60 s exposure times of *P. ostreatus* with various growth times compared to *T. versicolor*. The increased hydrophobicity of the composite surface is attributable to both the increased chitin content of the composite papers and their higher extractives content. Composite papers produced from fibres extracted from manure colonised with *T. versicolor* exhibited a 50% higher extractive content than *P. ostreatus* papers (1.2 wt% compared to 0.79 wt%, respectively) after 16 weeks resulting in a more hydrophobic surface and influencing the imbibition of the water droplet (Table 1). The hydrophobicity of fungal mycelium derived papers, which can exhibit water contact angles up to 106° can be attributed to naturally occurring compounds, such as lipid residues and hydrophobins present in the biomass.[21,28]

The considerably more hydrophobic nature of papers produced using lignocellulosic fibres extracted from manure after fungal colonisation, with θ similar to or exceeding those reported in the literature for (ligno)cellulose (29° to 88°),[48–50] crustacean chitin (24° to 55°)[23,51,52] and even neat chitin-β-glucan papers produced from fungal fruiting bodies (54 to 67°)[23] demonstrated the potential of the fungal modification of typically very hydrophilic natural materials to exhibit more

hydrophobic surface characteristics through colonisation and subsequent hybridisation with fungal biopolymers.

Conclusion

The potential for modification of lignocellulosic biomass using biological fungal growth stretches well beyond delignification and traditional biopulping practises, with the chitin-β-glucan networks of fungal mycelium offering unique opportunities to hybridise lignocellulosic fibres in papers and tune mechanical and surface properties. Delignification and hybridisation of lignocellulosic material extracted from elephant manure was achieved in as little as 4 weeks using *P. ostreatus* or *T. versicolor* fungal growth, resulting in considerably more hydrophobic surfaces of composite papers. Extended periods of fungal growth, spanning up to 16 weeks, facilitated considerable interfacing of cellulose microfibrils through nanoscale fungal chitin-β-glucan networks within the papers, resulting in significant improvements in tensile strengths and elastic moduli. These improvements were most notable in composite papers that were delignified and hybridised using *P. ostreatus* fungal growth. These findings highlight the missed opportunities in traditional bioprocessing and modification of lignocellulosic materials and demonstrate the considerable potential of the fungal biorefinery as an environmentally sustainable and as yet underexploited technology.

Experimental section

Materials

Trametes versicolor and *Pleurotus ostreatus* fungal cultures were purchased from Tyroler Glückspilze® Mushroom Production Center GmbH, Austria. The samples were supplied as mycelium on saw dust spawn in a plastic bag sealed with a filter patch. Subcultures were plated onto fresh sterile malt extract agar plates (Neogen, Michigan, USA) and incubated in darkness at 25 °C for 5 d prior to use as inoculum. Fresh elephant manure was provided by Vienna Zoo (Tiergarten Schönbrunn). According to the elephant keepers of Vienna Zoo the elephants mainly eat hay, shrubs, grass but also fruits and bread. Blackstrap molasses was purchased from Nortem Biotechnology (El Puerto de Santa Maria, Spain). NaOH (≥97.0%) and H_2SO_4 (95%) were purchased from Sigma-Aldrich and used as received. Sugar recovery standards (SRS) for carbohydrate analysis were prepared from L-(+)-arabinose (Merck 101492), D-(+)-galactose (Merck 3455), D-(+)-glucosamine hydrochloride (Sigma-Aldrich G4875), D-(+)-glucose (VWR 10117HV), D-(+)-mannose (Merck 4440), L-(+)-rhamnose (Merck R3875) and D-(+)-xylose (Merck 108689). Deionized water was used for all experiments.

Fungal growth on elephant manure

Fresh elephant manure (collected April 2019) was sterilised at 120 °C for 90 min in an autoclave and stored at −11 °C pending further use. The material was defrosted at RT prior to use and re-sterilised (120 °C, 90 min). Manure aliquots (3–4 g dry mass) of 30 ± 0.03 g were deposited into 90 mm Petri dishes, which were then inoculated using *P. ostreatus* and *T. versicolor*, respectively. 8 mm diameter inoculum disks were cut from solid tissue culture. Petri dishes were sealed with paraffin film and incubated in darkness at 25 °C for 4, 8, 12 and 16 weeks. A minimum of six Petri dish replicates were plated for each growth period. The dry mass, m, of each sample was determined both pre- and post-growth across triplicate samples using a micro-balance (Sartorius Cubis®) following an oven drying period of 24 h at 105 °C.

Extraction of natural polymers

Following the designated growth period, the colonised elephant manure material was blended for 3 min in 500 mL of water and the resulting suspension heated to 85 °C for 30 min. The suspension was then cooled to 25 °C and centrifuged for 10 min at 8500 rpm, before the supernatant was discarded, the residue resuspended in 500 mL of 1 M NaOH and reheated to 65 °C for 3 h. Washing and neutralisation was achieved by repeated centrifugation and redispersion in water until pH 7 was reached. The extraction yield ($\text{yield}_{\text{extraction}}$) was assessed based on the previously determined $m_{\text{post-growth}}$ and the dry mass of the material measured after extraction $m_{\text{post-extraction}}$ using eqn (1). Extracted material was stored at 4 °C pending further use. Total yield following both growth and extraction ($\text{yield}_{\text{total}}$) was then calculated using eqn (2). The use of these equations is further illustrated in Fig. 1.

$$\text{Yield}_{\text{extraction}}(\%) = \left(\frac{m_{\text{post-extraction}}(\text{g})}{m_{\text{post-growth}}\ (\text{g})}\right) \times 100 \quad (1)$$

$$\text{Yield}_{\text{total}}(\%) = \left(\frac{m_{\text{post-extraction}}(\text{g})}{m_{\text{pre-growth}}\ (\text{g})}\right) \times 100 \quad (2)$$

Preparation of composite papers

Extracted material was suspended in water (1.22 g, 0.4% w/v) and vacuum filtered (VWR 413, 125 mm qualitative filter paper, particle retention 5–13 μm) before being peeled and hot-pressed using 20 kN of force at 120 °C for 15 min to produce the final composite papers.

Morphological and chemical analysis of composite papers

Scanning electron microscopy (SEM) imaging of the composite papers was performed using a NeoScope™ JCM-7000 instrument (Jeol, Japan) and an accelerating voltage of 30 kV. All samples were coated (30 s, 30 mA) with gold prior to imaging (Fine coater JFC-1200, Jeol, Japan).

Elemental composition was determined for triplicate 2 mg samples using an EA 3000 CHNS-O Elemental Analyser (Eurovector, Italy) with a high temperature pyrolysis furnace (HT, Hekatech, Germany 2009).

Carbohydrate analysis was carried out by high performance anion exchange chromatography (HPEAC). 300 mg freeze dried samples were mixed with 3 mL of 72% sulfuric acid at 30 °C

for 60 min. The acid was then diluted with water to a concentration of 4% and the mixture placed in an autoclave at 121 °C for 60 min. HPEAC was performed on the previously diluted acid hydrolase using a chromatograph (Dionex ICS3000) equipped with a CarboPac PA20 column. Sugar Recovery Standards (SRS) were prepared and pre-treated at identical hydrolysis conditions prior to HPAEC analysis in order to analyse their recovery throughout the procedure.

The extractives content of the composite papers was determined using Soxhlet extraction. 0.5 g samples were dried at 105 °C for 12 h and weighed ($m_{\text{pre-hexane-extraction}}$). Dry samples were then extracted with 200 mL of hexane for 6 h in a 30 mL Soxhlet apparatus (4–5 cycles per h). Following the extraction, the samples were dried again at 105 °C for 12 h and weighed ($m_{\text{post-hexane-extraction}}$). The extractives content was calculated using eqn (3).

$$\text{Extractives content(wt\%)} = \left(1 - \frac{m_{\text{post-hexane-extraction}}\ (\text{g})}{m_{\text{pre-hexane-extraction}}\ (\text{g})}\right) \times 100 \quad (3)$$

Acid-insoluble lignin (AIL) was determined according to standard T222 om-02. Composite papers were ground to a particle size <1 mm (Universal mill, UM620, Karcher, Germany). Water content was evaluated using Karl Fischer titration (Mettler Toledo V20, Columbus, Ohio, USA). Extracted material was treated using the same procedure described above for sugar analysis (72% H_2SO_4, diluted to 4% and heated to 121 °C for 60 min). The mixture was filtered over a filter crucible (pore size 3) and the solid residue dried at 105 °C and weighed (m_{AIL}). Finally, the acid-insoluble ash content was determined by placing filtrates into a muffle furnace at 500 °C for 3 h (m_{ash}). A correction for ash contained in lignin was performed in accordance with standard T211 om-02 using eqn (4).

$$\text{AIL (wt\%)} = \frac{m_{\text{AIL}} - m_{\text{ash}}\ (\text{g})}{m_{\text{sample}}(\text{g})} \times 100 \quad (4)$$

Physical and mechanical properties of composite papers

The papers were cut into dog bone shaped specimens (type 1BA, ÖNORM EN ISO 527-2, 2012) using a Manual Cutting Press (Zwick ZCP 020). Specimens had a parallel width of 5 mm and an overall length of 75 mm. The thickness of each specimen was determined using a digital micrometer (AnyiMeasuring). Prior to mechanical testing, all samples were conditioned in the laboratory at standard conditions (23 ± 2 °C and RH 50 ± 3%) for at least 48 h. Tensile tests were performed using a dual column universal testing system (5969, Instron) equipped with a 1 kN load cell and a non-contact video extensometer (Gig ProE iMETRIUM). Specimens were fixed between metal clamps using blotting paper to avoid perforation of the samples. Testing velocity was 1 mm min^{-1} with gauge length set to 25 mm. The elastic modulus E was analysed in the linear elastic region as a secant between stress values separated by 0.2% strain. The tensile strength σ was calculated from maximum load and specimen cross-sectional area.

The paper skeletal density (ρ_s) was determined by helium gas displacement pycnometry (Micromeritics AccuPyc II 1340) with a chamber volume of 1 cm^3.

The paper grammage (G) (eqn (5)) was determined by dividing its mass (m) by the area of the specimens. The thickness of the papers d was measured using a digital outside micrometer (AnyiMeasuring), with 20 replicate measurements across the paper. d was used to calculate the envelope density ρ_e of the papers (eqn (6)). The porosity Φ was in turn calculated from the skeletal and envelope densities (eqn (7)).

$$G\ (\text{kg m}^{-2}) = \frac{m\ (\text{kg})}{\pi \times r^2\ (\text{m}^2)} \quad (5)$$

$$\rho_e\ (\text{kg m}^{-3}) = \frac{G\ (\text{kg m}^{-2})}{d\ (\text{m})} \quad (6)$$

$$\Phi(\%) = \left(1 - \frac{\rho_{\text{envelope}}(\text{kg m}^{-3})}{\rho_{\text{skeletal}}\ (\text{kg m}^{-3})}\right) \times 100 \quad (7)$$

Surface analysis of composite papers

Static contact angles of water droplets on the composite papers were determined using a drop shape analyser (DSA30, Krüss GmbH, Hamburg, Germany). The initial contact angle was recorded 5 s after dosing (2 μL drop of each test liquid), followed by measurements after 30 s and 60 s. At least 50 measurements at 10 individual sites were taken. R studio (v. 1.2.5019, RStudio Inc., Boston, MA, USA) was used for statistical analysis. The significance of the differences in contact angles was assessed using analyses of variance (ANOVA). In case of significant differences, a Tukey's HSD test was used ($p < 0.05$) to identify groups and means.

Conflicts of interest

There are no conflicts to declare.

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Environmental life cycle assessment of nano-cellulose and biogas production from manure

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ABSTRACT

Due to its unique properties, nano fibrillated cellulose (NFC) has been a popular topic of research in recent years. Nevertheless, literature assessing environmental impacts of NFC production is scarce, especially for using other starting materials than wood pulp. Hence, in this study, a new approach of cascaded use of manure to produce biogas and subsequently use the cellulose containing digestate for NFC production (manure scenario) is compared to the production from Kraft pulp from hardwood chips (wood chips scenario) via life cycle assessment (LCA). To produce comparable outputs (NFC and biogas) in both scenarios a typical Austrian biogas plant with maize silage and pig slurry as input material is included in the wood chips scenario. A proxy approach is used to upscale the manure scenario from laboratory to an industrial scale (except for the pulp to NFC step) to ensure comparability of both scenarios. The impact categories global warming potential (GWP), fossil resource scarcity, freshwater eutrophication, human toxicity, terrestrial acidification (TAP) and terrestrial ecotoxicity potential are analysed referring to the functional unit of 1 kg NFC. Results show that the manure scenario has at least 45% lower impacts in all assessed categories. GWP is 4.41 kg CO_2 eq./kg NFC in the manure and 9.74 kg CO_2 eq./kg NFC in the wood chips scenario. The transformation step from pulp to NFC is identified as environmental hotspot due to the high electricity demand in both scenarios. Results are additionally assessed only for the industrial scale part (includes biogas and pulp production). In the latter the main difference can be found in the substrate production. While it plays a subordinate role in the manure scenario (up to 8%) as manure is seen as a waste stream with no upstream environmental impacts attached, the production of maize silage is one of the hotspots in the industrial part in the wood chips scenario. This difference is especially prominent in TAP, where the substrate production is responsible for 91% of the 0.06 kg SO_2 eq. impact, which is tenfold the impact of the manure scenario. This underlines the issue of using energy crops as substrate in biogas plants. It also highlights the importance of further research of using waste streams as inputs for the electricity production and subsequent use in the pulp and paper industry. This LCA demonstrates that NFC production from manure is a sustainable alternative to the production from hardwood Kraft pulp.

1. Introduction

Nano fibrillated cellulose (NFC) is a unique new material with a wide range of potential applications such as paper making or in composites. In general, NFC is isolated from cellulose fibres and has fibril diameters below 100 nm (Josset et al., 2014; Li et al., 2013). In recent years NFC is widely researched because of its excellent chemical and mechanical properties such as high strength and modulus, biodegradability,

Abbreviations: CHP, combined heat and power; FEP, freshwater eutrophication; FRS, fossil resource scarcity; GWP, global warming potential; HCTP, human carcinogenic toxicity potential; HNCTP, human non-carcinogenic toxicity potential; LCA, life cycle assessment; NFC, nano-fibrillated-cellulose; PP, polypropylene; TAP, terrestrial acidification; TEP, terrestrial ecotoxicity potential.

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biocompatibility and non-toxicity (e.g. Gupta and Shukla, 2020; Pulidindi and Pandey, 2020).

The most common NFC production route is a mechanical treatment (e.g. homogenization or grinding) of wood pulp with an optional chemical pre-treatment (e.g. oxidation or carboxymethylation) with the usual starting material being wood pulp (Li et al., 2013). In 2017 the world total pulp production was 184.4 million tonnes with the largest share of 34.9% being produced in North America (CEPI, 2019). Forecasts predict that the pulp and paper consumption will grow steadily, particularly in Asia due to increasing living standards. Further, substantial climate change impacts can be attributed to the pulp and paper industry throughout the whole product life cycle, starting from harvesting the raw material, through the energy and water intensive production to the end of life of the products (Environmental Paper Network, 2018). Additionally, studies already reported that the transformation from pulp to NFC is responsible for major environmental impacts, especially due to the high electricity demand (Li et al., 2013; Turk et al., 2020).

To reduce the environmental impacts of the pulp and paper industry (and therefore as well for NFC production) the Environmental Paper Network (2018) suggests that alternatives for wood fibres should always be considered. Those include hemp, flax, bamboo, kenaf, and agricultural residues such as wheat straw and bagasse (Favero et al., 2019), but also manure as a by-product from farms.

Literature studying the environmental impacts of the NFC production is scarce, not to mention NFC production from alternative sources. Available publications are focusing on different production routes, starting materials (all studies use either wood or wood pulp, except for the study from Piccinno et al. (2018)) and include different life-cycle stages.

Li et al. (2013) combined a chemical pre-treatment (TEMPO-oxidation or chloroacetic acid etherification) with a mechanical disintegration method (sonication or homogenization), respectively and therefore compared four different laboratory fabrication routes by using delignified pulp as starting material. Results showed that the TOHO route (TEMPO-oxidation combined with homogenization) caused the least global warming potential (GWP) of 190 kg CO_2 eq./kg NFC compared to up to 1160 kg CO_2 eq./kg NFC. Nguyen (2014) assessed the total energy demand of an enzymatic and a carboxymethylation pre-treatment followed by homogenization and reported the enzymatic fabrication route to have the lowest energy consumption (90 MJ/kg); the carboxymethylation route the highest (1450 MJ/kg). Turk et al. (2020) also assessed the TOHO fabrication route in the laboratory but used thermo-groundwood as starting material and Soxhlet extraction for pulp. The GWP found by Turk et al. (2020) was between 770 and 814 kg CO_2 eq./kg NFC taking three different impact assessment methods (ILCD/PEF, CML 2001 and ReCiPe 2016) into account. Arvidsson et al. (2015) used elementary chlorine free sulfate, totally chlorine free sulfate, unbleached sulfate (baseline scenario), and chlorine bleached sulfite pulp as starting materials in the laboratory by using a mechanical approach with different pre-treatments: an enzymatic, a carboxymethylation pre-treatment which is similar to the chloroacetic pre-treatment used from Li et al. (2013) and one without pre-treatment at all. The GWP in the baseline scenario was ~100 kg CO_2 eq./kg NFC for the carboxymethylation route and for the enzymatic and the no pre-treatment route below 5 kg CO_2 eq./kg NFC (Arvidsson et al., 2015). These results are lower than the GWP stated by Li et al. (2013) with 190 kg CO_2 eq./kg NFC. Though, it must be noted that Li et al. (2013) assumed a higher input of chemicals in their pre-treatment process (Arvidsson et al., 2015). Sun et al. (2013) performed a life cycle assessment (LCA) using a different chemical-mechanical approach to produce NFC by assessing the environmental burdens of a combined wet disk milling and mild hot-compressed water process in the laboratory with a cradle-to-gate approach and compared it to the environmental performance of petroleum-based polymers like polypropylene (PP). Also, the GWP of NFC was between 1.26 and 3.68 kg CO_2 eq./kg NFC (the wet disk milling process contributing 57–73% to it), which is in the range of 1.84 kg CO_2 eq./kg PP (Sun et al., 2013) and is comparable to the findings of Arvidsson et al. (2015).

Just one paper was found in the industrial scale, which examined the NFC production from carrot pomace by developing a method with five steps to scale-up a laboratory process (Piccinno et al., 2018). The comparison of the up-scaled process with the lab-scale scenario showed a reduction in environmental impacts (factor 3 for the highest impact industrial scenario to factor 6.5 for the lowest impact scenario), which is in line with the potential reduction stated by Li et al. (2013).

Due to its relatively high cellulose content of up to 40% depending on the animal species (Meissner et al., 1990), manure as a by-product from farms can be used as resource for pulp and papermaking. In biogas production manure is widely used as substrate. Thereby energy and a fermentation residue containing valuable nutrients, which can be used as a fertilizer in agriculture are provided (Holm-Nielsen et al., 2009). A novel approach is to first anaerobically digest manure and produce biogas and subsequently use the cellulose-containing fermentation residue as a pulp resource, which is finally turned into NFC through a grinding step (Weiland et al., 2021). For further information on the novel fabrication route, as well as on chemical properties, see publication Weiland et al. (2021).

A recent review covering LCAs of NFC production points out that LCAs considering the industrial production scale are limited due to various challenges in e.g. data availability (Foroughi et al., 2021). Further, to the knowledge of the authors no LCA study exists at the industrial scale that covers a whole biorefinery, producing biogas from manure with subsequent use of the solid fermentation residue as raw material input to produce NFC. Hence, in this paper this novel fabrication route is examined with a life cycle assessment by using a cradle-to-gate approach to quantitatively assess environmental impacts and compare it to one of the most common fabrication routes usin g hardwood Kraft pulp as starting material. The study is based on the master thesis from Krexner (2020).

2. Material and methods

2.1. Life cycle assessment

The life cycle assessment is used as a methodical approach to assess two different NFC production routes. LCA is a well-established method to estimate the environmental impacts of a product or a service and is based on ISO standard 14040 (ISO, 2006a) and 14044 (ISO, 2006b). The applied software to perform the LCA is openLCA version 1.9 (Green Delta GmbH, 2018) with the Ecoinvent database version 3.6 (Wernet et al., 2016) and the impact assessment method ReCiPe2016 Midpoint (H) (Goedkoop et al., 2013; Huijbregts et al., 2017). Commonly a timeframe of 100 years is selected for this perspective (Goedkoop et al., 2013). The impact categories climate change (GWP), freshwater eutrophication potential (FEP), fossil resource scarcity (FRS), terrestrial acidification potential (TAP), human carcinogenic toxicity potential (HCTP), human non-carginogenic toxicity potential (HNCTP) and terrestrial ecotoxicity potential (TEP) were examined. As functional unit, which provides a reference to which all inputs, outputs and therefore results are referring to (ISO, 2006a), 1 kg of dry NFC is chosen. Although the examined system is a multi-output process providing biogas and NFC as products, the functional unit only refers to NFC since this provides the main benefit. Nevertheless, if systems are compared with each other by means of LCA, they must have the same overall benefits (Fleischer and Hake, 2002).

Although the biogas is not the main focus of this LCA, the biogas production portrays an integral part of the novel approach to produce NFC from manure. Furthermore, LCA aims to provide a clear and comprehensive picture of a system (Ekvall and Finnveden, 2001). For these reasons and with regards to ISO 14044 (ISO, 2006b) to avoid allocation and to guarantee a fair comparison, a common benefit with

the same amount of biogas and NFC is included in both the assessed systems. It also avoids producing scenarios with negative emissions, which are common for the use of credits, but often lacking practical viability.

Two scenarios are modelled (see section 2.2 and 2.3), comparing two pathways to produce NFC based on different raw material sources; each system encompasses a life cycle from raw material extraction to the final product provided at the factory gate. The novel approach to produce NFC from manure (manure scenario) is compared to a baseline scenario representing a status quo production of NFC based on wood pulp (wood chips scenario).

2.2. Manure scenario

The manure scenario is based on the novel approach of initial anaerobical fermentation of manure leading to biogas production and subsequently using the cellulose-containing fermentation residue as pulp resource, which is finally turned into NFC through a grinding step (Weiland et al., 2021). Elephant manure is used as a model system and extreme example in advance of further experiments with other more common manure types due to its high cellulose content of up to 40% (Meissner et al., 1990). It is assumed that all kinds of manure from livestock fed diets with high fibre contents e.g. horses, gestating sows or cattle could be used for this approach with only small adaptions needed in the chemical procedure. The method to produce NFC from herbivore manure is more closely described in Weiland et al. (2021). Only if the production route via elephant manure (with its extraordinary high cellulose content) is promising, it is reasonable to continue with livestock manure that has a lower cellulose content. Nonetheless, the product system is modelled and assumptions are made (e.g. transport distance for the manure) in such a way that these fit not only for the use of elephant manure, but generally for manure available in large quantities in Austria. In addition, the data based on elephant manure are used only for the calculation of biogas and methane production in the biogas plant and for the additional wastewater and inorganic solids arising with the use of this specific fermentation residue in the pulp production.

The novel fabrication route is currently only conducted in the laboratory and therefore has to be upscaled by using a proxy approach since the comparison between lab-scale data and industrial data (highly industrialised Kraft process; baseline scenario) does not lead to realistic results due to the optimized use of material, energy and other inputs at the industrial scale. Although the proxy approach is a method with an average low accuracy, it can be as accurate and faster as compared to other upscaling methods, such as process simulation or molecular structure models, if the proxy is well chosen. If the proxy data set is complete, this method provides a first estimation of environmental impacts (Parvatker and Eckelman, 2019). In this paper a biogas plant is used as proxy for the biogas production in the laboratory. A non-integrated pulp mill (i.e. the pulp and paper production take place at different sites), using softwood as starting material is the proxy for the pulp-like intermediate laboratory fabrication route. No accurate proxy was found in literature for the NFC production through mixing and grinding, though it can be assumed that the mixing step is already present in the industrial pulp production and is therefore not considered as a separate step in the industry scale model anymore. Therefore, the grinding step is modelled for both scenarios at the laboratory scale based on primary data for each scenario. This leads to the division in both scenarios into the industrial part that includes the biogas and pulp

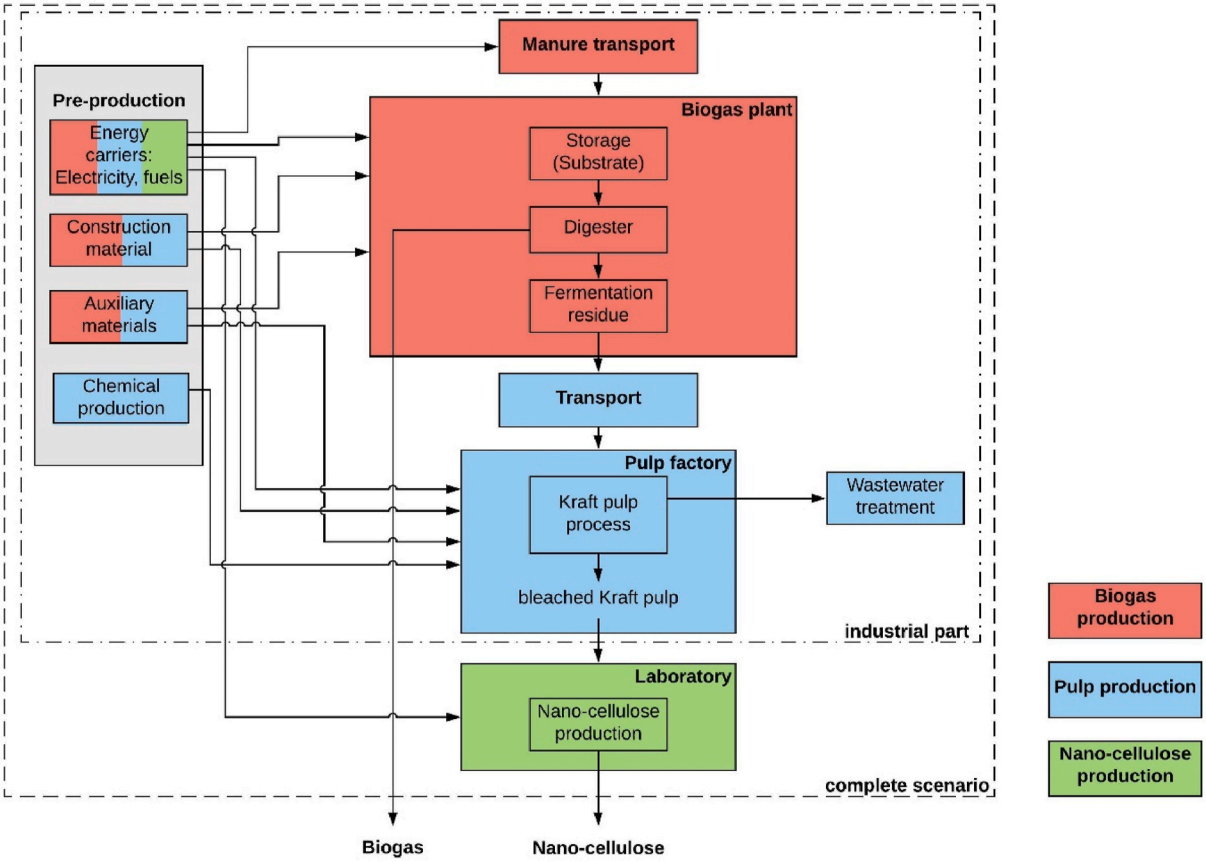

Fig. 1. System diagram of the manure scenario; the dashed line illustrates the system boundaries for the complete scenario; the processes upscaled to the industrial part are within the dot and dash line; the NFC production is at the laboratory scale; red processes are related to biogas production, blue processes to the pulp production, and green processes to NFC production. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

production at the industrial scale and the complete scenario, which includes the industrial part and the NFC production at the laboratory scale. The system diagram of the manure scenario is illustrated in Fig. 1; the dashed line marks the system boundaries of the whole product system while the dot and dash line illustrates the industrial part only.

Since a cradle-to-gate approach is applied the construction materials and associated transports for both the biogas plant and the pulp factory, as well as transport for all used chemicals are inside the system boundaries. Since elephant manure is considered as by-product or even waste and not as a primary benefit in elephant keeping and it has no market value in Austria, all environmental impacts are allocated to keeping these animals. Therefore, no upstream environmental burden is considered for elephant manure which is in line with ISO standards 14040 (ISO, 2006a). If other manure types would be used, they could also be considered as waste with no upstream emissions attached or environmental burdens of upstream emissions would mainly be allocated to the main products e.g. milk or meat.

The biogas plant with a lifespan of 15 years has a main digester and a fermentation residue storage with a volume of 2701 m^3, respectively and is modelled based on Kral et al. (2016). In this paper, in contrast to the publication of Kral et al. (2016), diluted elephant manure (from 22.4% dry matter content to 9.7% dry matter content to make it pumpable for the feeding and to reach better stirring during the wet fermentation (Fachagentur Nachwachsende Rohstoffe e.V., 2016)) is used as input material. The biogas and fermentation residue output, as well as the retention time (21 days) are based on laboratory experiments by Weiland et al. (2021), using elephant manure as input material for biogas production in order to simultaneously produce a starting material for NFC production with good mechanical properties and to receive a decent biogas output. The used elephant manure is transported over 4 km. This distance seems short but is feasible regarding the fact that elephant manure could be replaced for example by manure from other livestock in the European context, and the fact that biogas plants are built in particular proximity to animal husbandry. The loading and unloading of the manure and related emissions are assumed to be negligible based on the assumption that the used transporter is not started during these operations.

The dataset "sulfate pulp production, from softwood, bleached" from the Ecoinvent database (Wernet et al., 2016) is used for the non-integrated pulp mill. The fermentation residue is transported over 15 km to the pulp production site. Input material is adjusted to the fermentation residue from elephant manure, hence the liquid phase of the fermentation residue and some inorganic suspended solids are included as additional outputs since the fermentation residue is more fluid than the softwood normally used. During pulp production a by-product is produced that can be used as fertilizer, but the application of it is not within the system boundaries, as more research needs to be conducted about the nutrient-availability and potential risks to soil.

Since no accurate proxy was found in literature for the grinding step, it is modelled at the laboratory scale based on primary data by Weiland et al. (2021). Exact amounts of inputs and outputs of the biogas plant, the pulp production and for the NFC production from pulp through grinding can be found in Table 1 as life cycle inventory.

2.3. Wood chips scenario

The wood chips scenario serves as baseline scenario and consists of two big parts: the production of NFC and, to create equal benefits of both scenarios, also the production of biogas. Therefore, in order to assess comparable benefits, the same amount of biogas is included in the wood chips scenario that is produced in the manure scenario by the biogas plant fermenting manure referring to the functional unit of 1 kg NFC. Following the ISO standards 14044 (ISO, 2006b) the approach of utilization equality has to be prioritised over the use of allocation.

To model a typical Austrian biogas facility maize silage and pig slurry are chosen as input material reflecting the most common production path for biogas production in Austria (Energie-Control Austria, 2011; 2019; Hopfner-Sixt, 2005). Hardwood chips are used as input material, since although research is more and more focusing on other cellulose streams (e.g. food or pulp and paper mill wastes), the majority of commercial production plants still use wood as starting material for NFC production, e.g. Nippon Paper Industries Co. Ltd. (s.a.), Sappi Papier Holding GmbH (s.a.), Oji Holdings Corporation (s.a.) and Kruger Inc. (s.a.). In Fig. 2 the system diagram of the wood chips scenario is illustrated. The dashed line marks the system boundaries of the whole product system while the dot and dash line illustrates the industrial part (biogas

Table 1
Life cycle inventory data of the manure scenario.

Process	Input/ Output	Category	Probability distribution	mean	Unit	Standard Deviation	Reference
Biogas production	Input	Biogas plant		0.00000014	Number of items		Kral et al. (2016)
	Input	Electricity from grid	Logarithmic normal	1.05	kWh	1.00 (geometric standard deviation; GSD)	Kral et al. (2016)
	Input	Thermal energy	Logarithmic normal	0.97	kWh	1.00 (GSD)	Kral et al. (2016)
	Input	Elephant manure		36.22	kg FM		own calculation, based on Kral et al. (2016)
	Input	Water		47.09	kg		own calculation
	Output	Biogas	Normal	2.60	Nm^3	0.08	own calculation, based on Weiland et al. (2021)
	Output	Methane from fermenter leakages	Normal	2.07	g	0.000098	own calculation, based on Kral et al. (2016), Bachmaier (2012)
	Output	Fermentation residue		**78.71**	**kg**		own calculation, based on Weiland et al. (2021)
Pulp production	Input	Fermentation residue		78.71	kg		own calculation
	Output	Liquid phase of fermentation residue		75.21	kg		own calculation
	Output	Suspended solids		0.89	kg		own calculation
	Output	Sulfate pulp, from elephant manure, bleached		**1.43**	**kg**		Wernet et al. (2016)
NFC production	Input	Water		11.43	l		Weiland et al. (2021)
	Input	Pulp		1.43	kg		Weiland et al. (2021)
	Input	Electricity		8.57	kWh		Weiland et al. (2021)
	Output	Nano-cellulose		**1**	**kg**		Weiland et al. (2021)

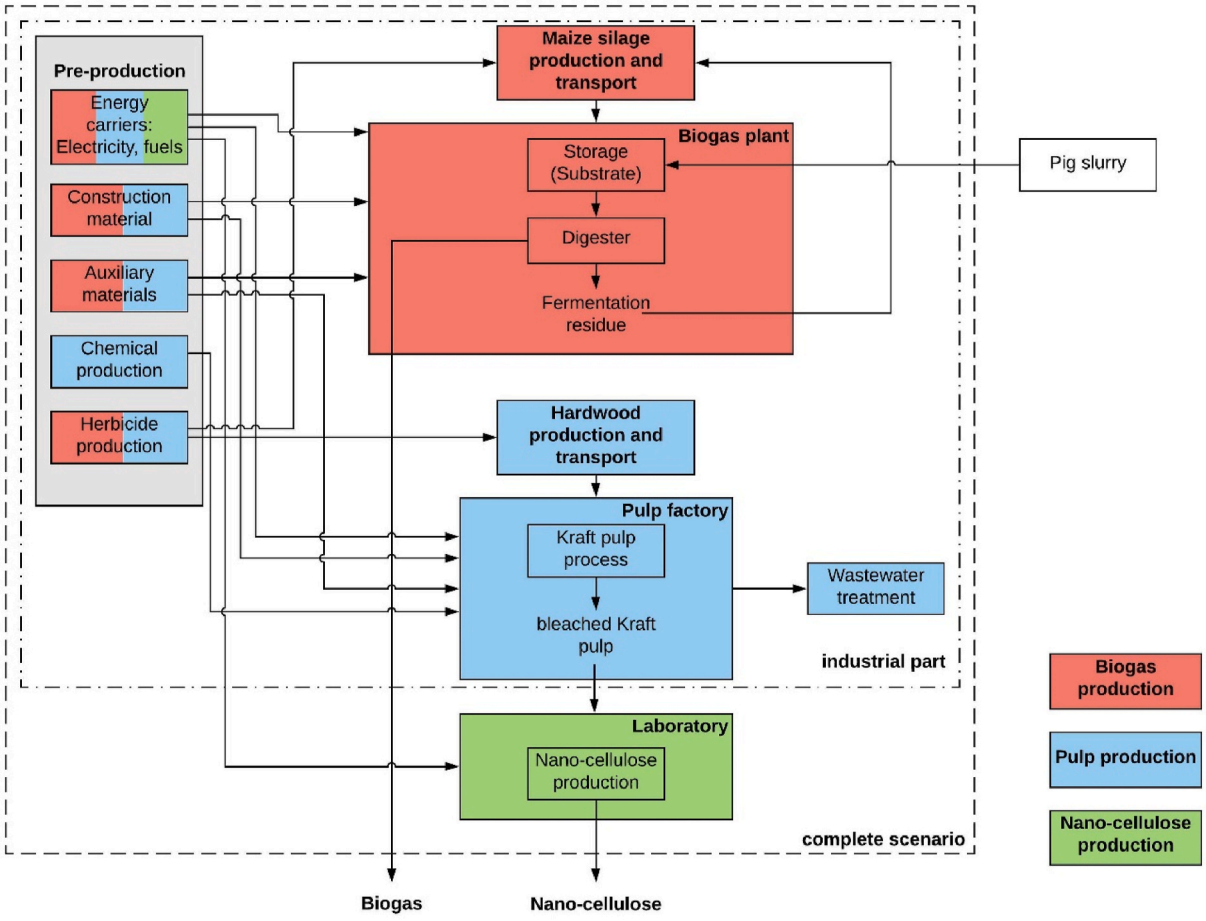

Fig. 2. System diagram of the wood chips scenario; the dashed line illustrates the system boundaries for the complete scenario; the processes upscaled to the industrial part are within the dot and dash line; the NFC production is at the laboratory scale; red processes belong to the biogas production, blue processes to the pulp production, and green processes to NFC production. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

and pulp production).

Again, a cradle-to-gate approach is applied including the upstream chains for both the production of the maize silage as input material for the biogas plant and forestry due to hardwood production as basis for the pulp production. Contrary to the manure scenario, in the wood chips scenario the upstream chain of the biogas plant input material is included, since maize silage has a market value in Austria and cannot be considered as waste. Still this system reflects the most likely production chain for biogas in Austria (Energie-Control Austria, 2011; 2019; Hopfner-Sixt, 2005), which is why it has to be used in the benchmark scenario.

For the maize silage production and transport, the process described in Kral et al. (2016) is used, which is based on the Ecoinvent process "maize silage production, Swiss integrated production, intensive" (Wernet et al., 2016), but the inputs, outputs and emissions are adjusted to Austrian conditions. The biogas plant with a main digester with a volume of 2701 m^3 and a lifespan of 15 years is again modelled based on Kral et al. (2016) with maize silage and pig slurry as input material. All further values (e.g. biogas output, retention time) regarding the biogas plant are assumed to reflect a typical Austrian biogas facility. The produced fermentation residue is used as fertilizer for maize and thus is encountered in the silage production by assuming that the nutrient requirements are roughly covered. Hence, there is no need for additional mineral fertilizer in this product system.

For the pulp production the Ecoinvent process "sulfate pulp production, from hardwood, bleached" is used and adjusted to Austrian conditions in terms of transport distances and electricity production by using the Austrian energy mix, which is mainly based on hydropower (Wernet et al., 2016).

Exact amounts of inputs and outputs of the biogas plant, the pulp production and for the NFC production from pulp through grinding can be found in the Supplementary Material Table S1 as life cycle inventory.

2.4. Statistical analysis

To test for significant differences between the scenarios, Monte Carlo simulations with 1000 iterations are conducted to assess overall uncertainty, followed by a Wilcoxon rank-sum test with a significance level of 0.05. In this paper 1000 iterations are chosen, since a higher number of runs will not go along with more precise results (Kral et al., 2016).

3. Results and discussion

LCA results were divided into two main sections: 1) the industrial part including all processes upscaled to industrial scale, i.e. transportation, biogas production and pulp production and 2) the complete scenario including the NFC production in addition to the industrial part. The results of the manure and the wood chip scenario for the industrial part and the complete scenario, respectively, are shown in the Supplementary Material Table S2. All values in Table S2 refer to the functional unit of 1 kg NFC and are representing the median value.

3.1. Relative environmental impact of the studied scenarios

The relative environmental impact of all examined impact categories for both the complete manure and wood chips scenario is shown in Fig. 3. For every impact category the absolute result values for the two scenarios are compared and the higher number is set to 100%. The lower number is shown as percentage share.

The manure scenario has a lower relative environmental impact in all examined impact categories. While the relative impact of the manure scenario ranges between 43% and 47% compared to the wood chips scenario in most of the impact categories (GWP, FRS, FEP, HCTP, HNCTP), the relative impact of the manure scenario in TAP is at a low level of 17% compared to the wood chips scenario. In the impact category TEP the manure scenario has the highest relative impact compared to the wood chip scenario with 55%.

The NFC production contributes between 35.11% (TEP) and 81.49% (FEP) to the total environmental impact of the manure scenario and between 21.79% (TAP) and 93.38% (FEP) of the wood chips scenario. Hence, the pulp to NFC production step is the main contributor to environmental impacts overall with 99.9% originating from the electricity demand.

The biogas production part in the manure scenario contributes less than 10% in all the examined impact categories except for GWP (10.07%) and TEP (14.24%), while in the wood chip scenario a biogas production share of under 10% can be found in FRS, FEP and HCTP. In TAP the highest share of 74.46% is observable. By assuming that the biogas production is a biological pre-treatment of lignocellulosic fibres to enable the production of NFC in the manure scenario, this part can be compared to other commonly used pre-treatments. The main difference is that by using anaerobic digestion as pre-treatment another useful product is provided in contrast to comparable methods. The production steps of NFC investigated in other studies, e.g. Turk et al. (2020), Li et al. (2013), Nguyen (2014) and Arvidsson et al. (2015) are usually a mix of chemical and mechanical treatments with the main contributor of environmental impacts being the pre-treatment part. This is not the case in this study where the biogas production plays a minor role in overall contribution.

The pulp production in both scenarios contributes less than 7.30% in all the examined impact categories except for TEP (manure scenario: 21.78%; wood chips scenario: 19%).

3.2. Contribution analysis

The contribution analysis divides the two major shares of the industrial part, namely the biogas and pulp production, into subsystems to explain the results in detail.

3.2.1. Biogas production

The biogas production is divided in the following subsystems and processes: 1) Biogas substrate production, which includes the transport of the manure and the supply with water for dilution for the manure scenario and in the case of wood chips scenario the cultivation and transport of maize and the transport for pig slurry; 2) Biogas plant construction: Construction materials and the needed transport to the biogas plant site; 3) Electricity and heat demand.

3.2.1.1. Biogas substrate production. In the manure scenario the substrate production and transport play subordinate role in TEP (8.06%) and HCTP (6.52%), respectively and are negligible in HNCTP (2.62%), TAP (2.19%) and FEP (1.82%), as can be seen in Fig. 4. This is due to the assumption that elephant manure is considered waste with no upstream environmental burden. In contrast, the production of maize silage, which is next to pig slurry the sole substrate for the biogas plant in the wood chips scenario, is one of the hotspots in the environmental impacts in all categories by having the highest or second highest share of impacts in the industrial part. Especially in TAP, the substrate production has a share of 91.42% (0.05 kg SO_2 eq./kg NFC) of the industrial part due to the fertilisation with liquid fermentation residue using a vacuum tanker and the occurring ammonia emissions as can be seen in Fig. 4. This source and the use of heavy machinery are accountable for the quite high shares compared to the manure scenario for HNCTP (42.50%; 0.89 kg 1,4-DCB/kg NFC), TEP (42.42%; 3.33 kg 1,4-DCB/kg NFC), GWP (28.97%; 0.44 kg CO_2 eq./kg NFC), FRS (24.63%; 0.09 kg oil eq./kg NFC) and HCTP (13.96%; 0.02 kg 1,4-DCB/kg NFC). Concerning HNCTP, especially the emission of zinc into the ground water and soil has a great impact. For FEP (19.40%, 0.0002 kg P eq./kg NFC) the highest impact comes from phosphorus emissions into water due to the maize silage production.

The big difference between the two scenarios is due the cultivation of maize and therefore the use of heavy machines, pesticides and fertilisation with fermentation residue, which must be considered in the wood chips scenario. This highlights the issue of energy crops produced exclusively for energy production, additionally to the competition with food production. This competition can be avoided by using residues, such as manure (Tilman et al., 2009). Elephant manure should be considered as an extreme example for alternative feedstock in the pulp and paper industry to demonstrate that NFC can indeed be produced from fermentation residue, but it could be replaced in Europe for instance by manure from livestock fed diets with high fibre contents e.g. horses, gestating sows or cattle. By using livestock manure as input

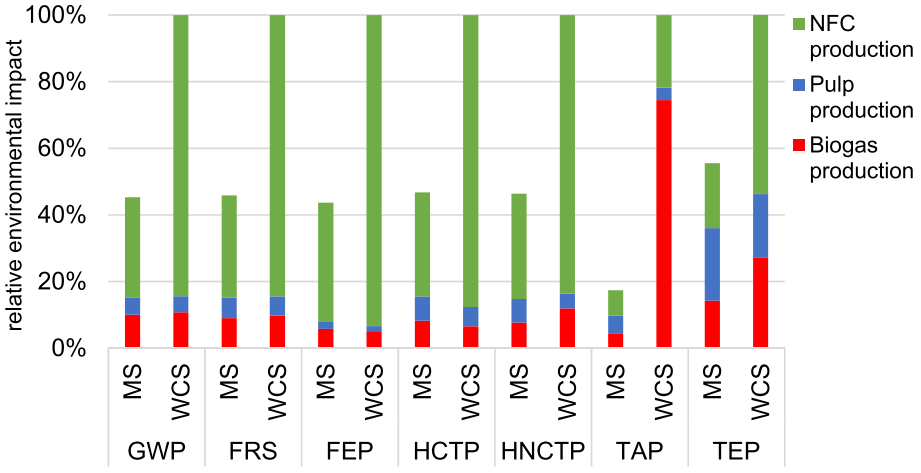

Fig. 3. Relative environmental impacts of the examined impact categories for the complete process chains of both scenarios; MS … manure scenario; WCS … wood chips scenario.

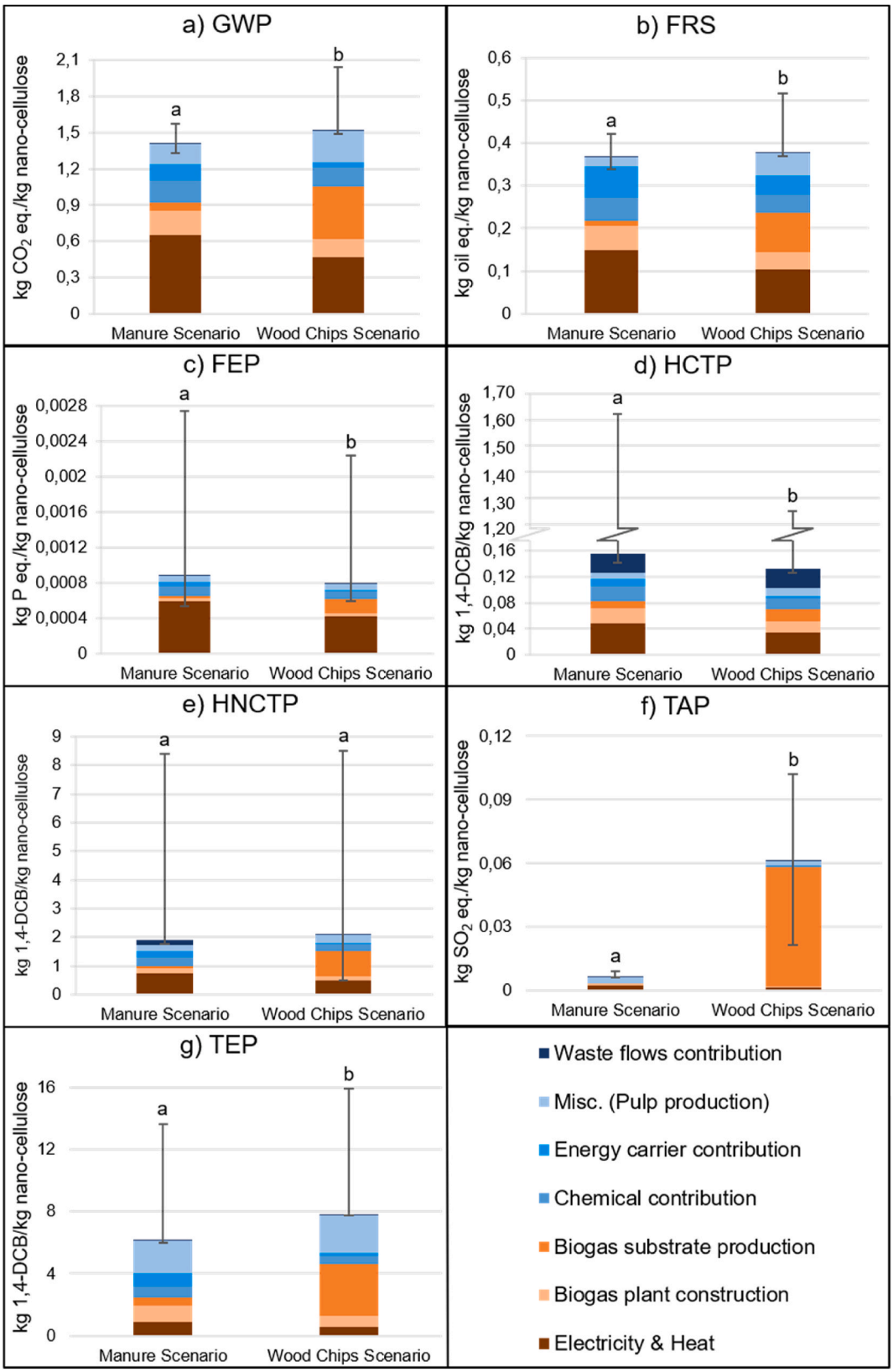

Fig. 4. Detailed contribution analysis of both scenarios for the assessed impact categories: a) GWP b) FRS c) FEP d) HCTP e) HNCTP f) TAP g) TEP; blueish shades is the impact related to pulp production, reddish shades the impact related to biogas production; error bars show the 5% and 95% interpercentile range of indicator's probability distribution function, based on 1000 Monte Carlo runs; lower-case letters represent significant differences. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

material, the upstream chain would have to be considered. In this case, the main emissions would be allocated to the products milk or meat. Leip et al. (2019) recently proposed a new method for allocation of environmental burdens of manure which should be seen as co-product as long as it is put to an appropriate use. In their two case studies they reported economic allocation factors of 18% and 6% for manure.

Overall, the results in this work are in line with the findings from Boulamanti et al. (2013) who stated that the cultivation of substrate is generally one of the main contributors to environmental impacts. Alike, Zhang et al. (2013) and Kral et al. (2016) emphasized that fertilisation with fermentation residue is one of the main sources of environmental impacts.

3.2.1.2. Biogas plant construction. The biogas plants in both scenarios consist of the same construction materials, but due to the lower yield of producing NFC from manure more pulp and therefore a higher share of the biogas plant is needed per functional unit. Therefore, the absolute values of the impact of the biogas plant construction is in all the assessed impact categories higher in the manure scenario than in the wood chips scenario. The relative impact of the industrial part in the manure scenario is the highest in TEP (17.15%) followed by HCTP (15.49%) and FRS (15.02%) and by far the smallest in FEP (4.56%). The relative impact in the biogas production in the wood chips is the highest in HCTP (13.96%) followed by FRS and FEP with 10.25%, respectively and the smallest in HNCTP (6.34%) (Fig. 4).

From the construction materials concrete, mastic asphalt, reinforcing steel and crushed rocks, concrete has the biggest overall impact over the assessed categories (GWP, FRS, HNCTP, HCTP, TAP) followed by mastic asphalt (GWP, FRS, HNCTP, FEP). Reinforcing steel is a major contributor to FEP and HCTP, while the use of crushed rocks affects only TEP. This is in line with findings of Kral et al. (2016).

3.2.1.3. Electricity and heat demand. The electricity and heat demand are one of the hotspots of environmental impacts overall in the industrial part. They are responsible of the highest or second highest share to almost all impact categories in both scenarios, except for TEP for both scenarios and TAP in the wood chips scenario. In these the substrate production is of major importance.

In the manure scenario the highest share from the electricity and heat demand with around two thirds is in FEP (64.70%; 0.0006 kg P eq./kg NFC) followed by shares of 46.28% (GWP: 0.65 kg CO_2 eq./kg NFC), 40.67% (FRS: 0.15 kg oil eq./kg NFC), 39,07% (HNCTP: 0.74 kg 1,4-DCB/kg NFC) and 38,08% (TAP: 0.0025 kg SO_2 eq./kg NFC). The lowest contribution can be found to HCTP (31.01%; 0.048 kg 1,4-DCB/kg NFC).

Around half of the environmental impact in the industrial part of the wood chips scenario is originating from the electricity and heat demand in FEP (49.54%; 0.004 kg P eq./kg NFC), while it contributes between a third to a quarter to GWP (30.64%; 0.46 kg CO_2 eq./kg NFC), FRS (27.76%; 0.10 kg oil eq./kg NFC), HCTP (26.06%; 0.034 kg 1,4-DCB/kg NFC), and HNCTP (24.19%, 0.51 kg 1,4-DCB/kg NFC) (Fig. 4).

3.2.2. Pulp production

The pulp production is divided in the following subsystems: 1) Waste flows: Including the waste management of green liquor dregs, waste wood, limestone residue, sludge from the pulp production, inert and municipal waste and waste mineral oil; 2) Chemicals: Supply of all the needed pulping chemicals including transport; 3) Energy carrier: Including the use of heavy and light fuel oil, natural gas and electricity; 4) Miscellaneous sources: Construction of the pulp factory (including the wastewater treatment facility and wastewater treatment process) for both scenarios, transports of the fermentation residue to the pulp site for the manure scenario and for the wood chips scenario also the cultivation and transport of the pulpwood.

It needs to be pointed out, that the discussion about the pulp production is limited since the processes of Kraft pulp production from softwood and hardwood are chosen as proxies based on Ecoinvent processes. Therefore, the analysis is limited by the data depth of these Ecoinvent processes.

Further, in this study hardwood chips for NFC production are chosen as benchmark system, since wood is currently mostly used in commercial production plants as starting material. Nevertheless, research is more and more focusing on other cellulose-containing streams, such as agricultural and forestry residues, which are already used occasionally. Hence, in future studies the benchmark system could change.

3.2.2.1. Waste flows. The contribution of the waste flows is negligible in the industrial part for GWP, FRS, FEP, TAP and TEP in both scenarios and only in the wood chips scenario also for HNCTP. Meanwhile, in the manure scenario the waste flows have a share of 8.30% with especially the disposal of wood ash mixture related to emissions of zinc and cadmium to agricultural soils playing a major role. In HCTP waste flows contribute 0.03 kg 1,4-DCB/kg NFC (manure scenario: 18.95%, wood chips scenario: 22.35%, respectively) to the environmental impact of the industrial part in both scenarios (Fig. 4). The main contributor is the waste disposal of green liquor dregs, related to chromium emissions into ground and surface water.

Similar numbers for HCTP are stated from González-García et al. (2011) who stated this high impact is coming from the disposal of wood ashes and green liquor dregs on landfills, which is in line with the results of this paper.

3.2.2.2. Chemicals. The higher contributions of chemicals and energy carriers to the industrial part of the manure scenario are due to the worse yield when producing nano-cellulose from manure compared to wood chips. Therefore, more pulp is needed which leads to a higher contribution from these two processes (Fig. 4).

The production and provision of sodium chlorate and sodium hydroxide has the biggest environmental impact overall in the assessed impact categories with the lowest share in total environmental impacts from chemical contributions of around 30% (sodium chlorate in HNCTP) up to over 50% (sodium chlorate in HCTP and sodium hydroxide in FRS). In GWP and FEP over 75% of the total environmental impact from chemical contribution is originating from the already mentioned chemicals and oxygen provision.

Other studies found the impact originating from chemicals also as one of the main contributors to pulp production (Corcelli et al., 2018; Ghose and Chinga-Carrasco, 2013; González-García et al., 2011).

3.2.2.3. Energy carriers. In HCTP the environmental impacts originating from energy carriers are around three times higher in the manure scenario compared to the wood chips scenario and in HNCTP around four times higher (Fig. 4). This can be justified by the fact that more pulp is needed in the manure scenario to produce the same amount of NFC. The biggest contribution to overall environmental impacts originating from energy carriers are related to natural gas and heavy fuel oil provision.

3.2.2.4. Miscellaneous sources. The miscellaneous sources share from the pulp production includes for both scenarios the overall construction of the pulp factory, for the manure scenario transports of the fermentation residue and for the wood chips scenario also the production and transport of pulp wood. Taking this into account, the higher absolute contributions of the miscellaneous sources part in the wood chips scenario can be explained.

In GWP and FRS the pulpwood production is the main contributor to the environmental impacts in the wood chips scenario (in total: 0.26 kg CO_2 eq./kg NFC; 0.05 kg oil eq./kg NFC, respectively) while in both scenarios in HCTP, HNCTP and TEP the construction of the pulp factory is the main contributor. The miscellaneous sources share is negligible in

FEP in both scenarios while only being negligible in TAP in the wood chips scenario. In the manure scenario of the latter category, it plays a major part with a share of 37.77% in the environmental impact of the industrial part (Fig. 4). This big difference can be explained by the fact that the substrate production in the wood chips scenario has such a tremendous impact on TAP that compared to it all other processes seem negligible. If the substrate production would be the same as in the manure scenario, the miscellaneous part would have around the same impact in both scenarios.

3.2.3. NFC production

The last step from pulp to NFC by grinding is found to be a hotspot in every of the assessed impact categories except for TAP and TEP as shown in Fig. 3.

In the complete production chain of the manure scenario the highest share of environmental impacts originating from NFC production is found in FEP with 81.49% followed by around 65% in the impact categories GWP, FRS, HCTP and HNCTP. The lowest shares are found in TAP (44.17%) and TEP (35.11%). In the wood chips scenario, the shares are even higher with the highest one also in FEP with 93.38% followed by around 85% in GWP, FRS, HCTP, HNCTP. The smallest shares of around a half to a quarter of the overall environmental impacts are found in TEP (53.74%) and TAP (21.79%).

In both scenarios and all the impact categories the main contributor to the NFC step is the electricity consumed (99%), while the water input is found to be negligible. The Austrian energy mix from the Ecoinvent database is used and for GWP, FEP, HNCTP the highest share of this part stems from electricity imports from Germany and the Czech Republic while for TAP the main share is originating from copper production and secondly from electricity imports from above-mentioned countries.

Even if this rather green energy mix is used, environmental impacts rise along with the amount of consumption. This is in line with other studies such as Turk et al. (2020), Li et al. (2013) and Arvidsson et al. (2015). The treatment of the NFC production is higher in this paper for the manure scenario than in the study from Arvidsson et al. (2015) due to the higher electricity demand.

Sun et al. (2013) estimated GWP of NFC production to be used as reinforcement in polypropylene with 1.2–3.7 kg CO_2 eq./kg NFC. Although, the impact of the manure scenario to GWP is with 4.41 kg CO_2 eq./kg NFC higher, this can be explained to the multi-output process delivering two products with market value: nano-cellulose and biogas.

The findings of Turk et al. (2020) who used thermo-groundwood as starting material and a Soxhlet extraction process in the laboratory are two magnitudes higher in GWP, FEP; HCTP and TAP and even three times higher in HNCTP. This is due to the high chemical demand for Soxhlet extraction step. In the terrestrial ecotoxicity potential the impact in their study is lower with 5.60 kg 1,4-DB eq./kg NFC than compared to the manure scenario with 9.46 kg 1,4-DB eq./kg NFC. This can be attributed to the high energy carrier contribution to the environmental impacts in the manure scenario. In general, the results of Turk et al. (2020) showed that the final step of turning pulp into NFC is the main contributor to overall environmental impacts as is stated in this study.

3.3. Sensitivity analysis

In the following section an attempt is shown to assess potential emissions savings of an upscaled grinding step and the influence on overall results. In a second sensitivity analysis the assumption of a combined production and use of biogas is tested by including a potential combined heat and power (CHP) unit which is supplied with the produced biogas and delivers enough off-heat to fully cover the biogas plants heat demand.

3.3.1. NFC production in the industrial scale

The grinding step, for which no proxy was found, is upscaled in this sensitivity analysis. Turk et al. (2020) and Piccinno et al. (2018) stated that the environmental impacts could be reduced by a factor 3 to 6.5, hence the input data for the grinding step is decreased by the mean of these factors (4.75). It must be taken into consideration that just the input material (pulp, water, electricity) is included in this analysis, but no building or machine that would be necessary. Also, no probability distribution is used for this sensitivity analysis.

In both scenarios a reduction of environmental impacts of the NFC production between 78.96% and 87.91% was found. Since the manure scenario offers a new route to NFC production, a great deal of attention was paid to the possible reduction of environmental impacts in an industrial production site. The highest reduction of the contribution is obtained in the human carcinogenic toxicity potential with a reduction of 87.91%. Likewise, in all other categories a reduction between 80.00% and 86.91% can be achieved (Fig. 5). This is possible due to the lower electricity demand for the NFC production since this process contributes the most to this step.

These results emphasise the importance of analysing the whole product system at the same scale – if data are available - which is also pointed out by Turk et al. (2020) and Piccinno et al. (2018).

3.3.2. Biogas plant heat demand covered by CHP unit off-heat

In this sensitivity analysis the assumption is made that a combined heat and power plant (CHP unit) is installed at the site of the biogas plant site for both scenarios and the heat produced is used to cover the heat demand of the biogas plant (mainly fermenter heating). It is assumed that the heat demand is fully covered by the off-heat of the CHP unit, which makes the use of heat from the grid unnecessary.

This leads to a reduction in the complete manure scenario of under 2% in FEP, HCTP and HNCTP. The decrease in environmental impact is slightly higher in TEP (3.85%), FRS (4.59%) and GWP (5.53%) with the highest decrease occurring in TAP with a reduction of 10.33% (0.0014 kg SO_2 eq./kg NFC). By just referring to the industrial part the reduction in TAP is even higher (21.74%). This can be justified by the fact that the heat and energy demand have the biggest share in this impact category in the industrial part with 38.08%.

In the complete wood chips scenario, the reductions are between 0.41% and 1.66%. The highest reduction is obtained in the global warming potential with 0.16 kg CO_2 eq./kg NFC which amounts to a reduction of 10.36% in the industrial part.

3.4. Theoretical potential of cattle manure

In the European context elephant manure is not available in large quantities, but cattle manure is. Since the assumption is made that every manure from livestock fed diets with high fibre contents e.g. horses, gestating sows or cattle could be used, the potential of cattle manure is calculated. It is assumed that per cattle and year around 9.32 t of manure (Bayerische Landesanstalt für Landwirtschaft, 2019) with a dry matter content of 23% (Landwirtschaftskammer Nordrhein-Westfalen, 2014) arise and the same amount of both cattle and elephant manure is needed for NFC production. For the case of Austria, 1,846,000 cattle (Statistik Austria, 2021) are held, which leads to a theoretical potential of 549, 425 t NFC/year.

4. Conclusions

The utilization of manure (considered as waste stream with no environmental impacts attached to its production) as starting material for the subsequent production of biogas and NFC will drastically reduce the environmental impacts in all examined impact categories compared to the production of NFC from Kraft wood pulp. Further, this fabrication route is a multi-output process, which not only provides NFC but also energy in the form of biogas.

The main contributor to environmental impacts in both investigated scenarios and for all assessed impact categories is the NFC production from pulp by grinding, which is modelled based on small scale

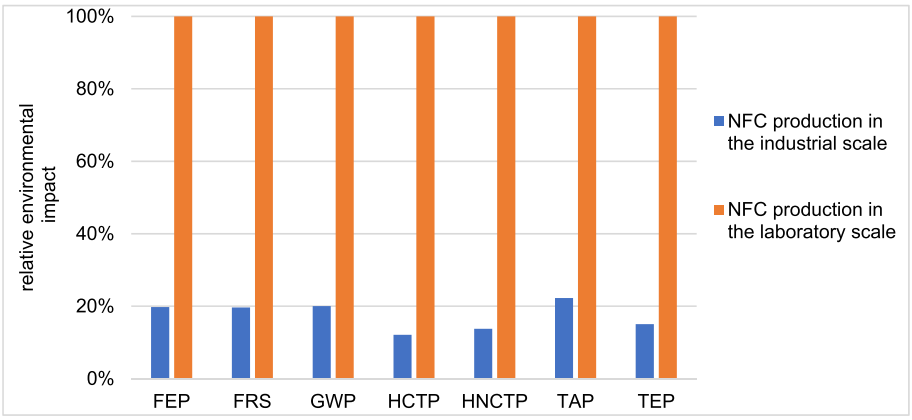

Fig. 5. Comparison of the relative environmental impacts of the NFC production in the manure scenario at the industrial and the laboratory scale for all examined impact categories.

laboratory data. This is in line with other studies. A sensitivity analysis demonstrates that upscaling this step to industrial scale can reduce environmental impacts of this process up to 87.91% due to energy savings related to the use of more efficient machinery and technologies.

Overall, this work demonstrates that NFC production from anaerobically fermented manure as starting material is a sustainable alternative to the production from hardwood Kraft pulp. Further, as results are promising more research is suggested.

5. Future perspectives

One of the biggest limitations of this work is the use of proxies to receive data for the life cycle inventory of the biogas and pulp production steps. Although, the here presented results are based on valid assumptions for the new fabrication route, data should be replaced as soon as possible with more accurate data of a real production process of a potential pilot plant. The limitations underline that more research is needed.

As elephant manure will be only rarely used as starting material in a European context, it indicates the potential of the use of alternative feedstock such as manure from horses, cattle or sows in the pulp and paper industry, emphasizing the need of further research. Hence, a life cycle assessment with widely available manure in Europe should be conducted. Further, the chemical treatment of pulp from manure needs improvement as well as the embedding of the production site in a practical setting with short transport distances for manure, using by-products from the pulp production as fertilizer and closing nutrient cycles.

To receive a more holistic picture, LCA results should be combined with other sustainability assessment methods, such as various exergy assessment accommodating environmental as well as economic issues (Rosen, 2018) or a multi criteria analysis.

Finally, as results are promising, especially with regards to the high added value of using manure as a waste stream with no upstream environmental impacts as starting material for energy and material exploitation, further investigations assessing policy implications, such as incentives or fundings, are suggested. Especially, to implement requirements of the EU green deal (European Commission, 2019) and to strengthen circular economy perspectives of renewable resources as demanded by the Federal Ministry for Climate Action, Environment, Energy, Mobility, Innovation and Technology (BMK, 2021).

Author contributions

Conceptualization: Alexander Bauer, Iris Kral, Alexander Bismarck; Investigation: Iris Kral, Theresa Krexner, Kathrin Weiland; Methodology: Iris Kral, Theresa Krexner; Project administration: Alexander Bauer, Iris Kral; Supervision: Andreas Gronauer, Alexander Bauer, Iris Kral; Roles/Writing - original draft: Iris Kral, Theresa Krexner; Writing - review & editing; Alexander Bauer, Andreas Gronauer, Kathrin Weiland, Alexander Bismarck, Andreas Mautner, Werner Zollitsch, Francisco Medel-Jiménez

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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.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2022.115093.

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Supplementary Material

Table S1. Life cycle inventory data of the wood chips scenario

Process	Input/ Output	Category	Probability distribution	mean; min-max	Unit	Standard Deviation	Reference
Biogas production	Input	Biogas plant		0.00000009	Number of items		(Kral et al., 2016)
	Input	Electricity from grid	Logarithmic normal	0.70	kWh	1.00 (geometric standard deviation; GSD)	(Kral et al., 2016)
	Input	Thermal energy	Logarithmic normal	0.64	kWh	1.00 (GSD)	(Kral et al., 2016)
	Input	Substrate - Maize silage		14.87	kg FM		own calculation, based on Kral et al. (2016), Fachagentur Nachwachsende Rohstoffe e.V. (2016)
	Input	Substrate - Pig slurry		6.24	kg FM		own calculation, based on Kral et al. (2016), Fachagentur Nachwachsende Rohstoffe e.V. (2016)
	Output	Biogas	Triangular	**min: 2.94 mod: 3.11 max: 3.27**	**Nm³**		own calculation, based on Kral et al. (2016), Fachagentur Nachwachsende Rohstoffe e.V. (2016)
	Output	Methane from fermenter leakages	Triangular	min: 2.04 mod: 2.18 max: 2.26	g		own calculation, based on Fachagentur Nachwachsende Rohstoffe e.V. (2016), Bachmaier (2012)
	Output	Fermentation residue		17.26	kg		own calculation, based on Kral et al. (2016), Fachagentur Nachwachsende Rohstoffe e.V. (2016)
Pulp production	Input	Biogas	Triangular	min: 2.94 mod:3.11 max: 3.27	Nm³		own calculation
	Output	Sulfate pulp, from hardwood wood chips, bleached		**1.19**	**kg**		(Wernet et al., 2016)
NFC production	Input	Water	Uniform	min: 59.74 max: 82.44	l		(Weiland et al., 2021)
	Input	Pulp		1.19	kg		(Weiland et al., 2021)
	Input	Electricity	Uniform	min: 21.74 max: 25.57	kWh		(Weiland et al., 2021)
	Output	Nano-cellulose		**1**	**kg**		(Weiland et al., 2021)

Table S2. Life cycle impact assessment results of producing 1 kg of NFC of the complete scenario and the industrial part only, respectively including the 5th and 95th percentile for both the manure and the wood chips scenario

	Impact category	Manure scenario			Wood chips scenario			Unit [per 1 kg NFC]
		Median	5th percentile	95th percentile	Median	5th percentile	95th percentile	
Complete scenario	GWP	4,41	3,87	5,12	9,74	8,35	11,62	kg CO_2 eq.
	FRS	1,11	0,96	1,31	2,42	2,04	2,91	kg oil eq.
	FEP	4,85E-03	2,48E-03	0,01	0,01	4,95E-03	2,92E-02	kg P eq.
	HCTP	0,46	0,29	7,02	0,98	0,52	17,31	kg 1,4-DCB
	HNCTP	5,92	4,12	30,22	12,77	8,25	66,96	kg 1,4-DCB
	TAP	0,01	0,01	0,02	0,08	0,04	0,12	kg SO_2 eq.
	TEP	9,46	9,46	21,94	17,05	16,20	40,38	kg 1,4-DCB
Industrial part	GWP	1,43	1,33	1,58	1,54	1,49	2,04	kg CO_2 eq.
	FRS	0,37	0,34	0,42	0,38	0,37	0,52	kg oil eq.
	FEP	9,10E-04	5,60E-04	2,70E-03	8,50E-04	6,4E-04	2,30E-03	kg P eq.
	HCTP	0,16	0,14	1,62	0,13	0,13	1,25	kg 1,4-DCB
	HNCTP	1,89	1,77	8,39	2,09	0,50	8,51	kg 1,4-DCB
	TAP	6,49E-03	6,40E-03	9,1E-03	0,06	0,02	0,10	kg SO_2 eq.
	TEP	6,12	6,27	13,59	7,84	7,80	16,00	kg 1,4-DCB

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