



SYMSITES

High Value New Resources validation

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List of Abbreviations and Acronyms

Abbreviation	Definition
AATCC	American Association of Textile Chemists and Colorists
AC	Adsorption Column
AF_1	Antifungal formulation 1
AnMBR	Anaerobic membrane bioreactors
ATCC	American Type Culture Collection
BW	Biowastes
CG	Condensable Gases
CO	Cotton
D2.5	Deliverable 2.5
D2.6	Deliverable 2.6
D4.4	Deliverable 4.4
DME	Dimethyl Ether
GC-MS	Gas Chromatography-Mass Spectrometry
HVNR	High Value New Resource
KPI	Key Performance Indicators
NaOH	Sodium Hydroxide
NRW	Non-Recyclable Waste
PES	Polyester
PL	Pyrolytic Liquids
WP	Work Package



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1 ABSTRACT

This deliverable validates the production of high-value resources (HVNR) from waste using integrated technologies such as AnMBR and pyrolysis. It demonstrates the feasibility of generating hydrogen, biogas, biofertilizers, reclaimed water, and platform chemicals within a circular economy framework.

The results show strong energy recovery potential, regulatory compliance of sludge for agricultural use (with required hygienization), and the reuse of treated water in different sectors, although some limitations remain, particularly regarding turbidity and stability.

Industrial validation confirms that treated wastewater can be used in textile dyeing with negligible quality differences compared to conventional water.

Overall, the system is technically viable and sustainable, but further optimization and the resolution of regulatory constraints are needed to enable large-scale implementation.

2 EXECUTIVE SUMMARY

This deliverable presents the validation of High Value New Resources (HVNR) generated within the SYMSITES project through the integrated treatment of municipal, industrial, and organic waste streams across different European ecosystems. The work demonstrates the technical feasibility of transforming waste into valuable products by combining advanced technologies such as anaerobic membrane bioreactors (AnMBR), pyrolysis, membrane filtration, and biological processes within a circular economy framework.

The results confirm a significant potential for energy recovery, with methane production reaching approximately 2,351 m³ CH₄ per day, equivalent to around 23.5 MWh/day, and its subsequent conversion into renewable hydrogen through advanced reforming technologies. In parallel, the project validates the use of anaerobic sludge as a biofertilizer, showing high organic matter content and heavy metal concentrations well below regulatory limits, although highlighting the need for a hygienisation step to ensure microbiological safety prior to agricultural application.

The quality of treated wastewater has been assessed for multiple reuse scenarios, demonstrating compliance with high standards for urban applications and suitability for agricultural irrigation, while also identifying certain limitations related to turbidity and operational variability that require further optimisation. Reuse scenarios at regional scale confirm that a significant volume of reclaimed water can be effectively integrated into urban services and agricultural systems, contributing to resource efficiency and water savings.

Industrial validation activities further demonstrate the practical applicability of the developed solutions. In particular, the use of treated wastewater in textile dyeing processes at pre-industrial and industrial scale shows minimal colour differences compared to conventional water, confirming that process water substitution is feasible without compromising product quality. Additional valorisation routes, such as the recovery of platform molecules (e.g. benzoic acid), the production of biochar, and the validation of bio-based products for cosmetic and agricultural applications, highlight the versatility and added value of the system.



Overall, the deliverable confirms the robustness and multifunctionality of the SYMSITES approach, demonstrating its alignment with circular economy principles through resource recovery, waste minimisation, and industrial symbiosis. However, it also identifies key challenges related to process stability, energy consumption, regulatory constraints, and large-scale implementation, indicating the need for further optimisation and integration efforts to enable full industrial deployment.

3 OBJECTIVES

The main objective of Deliverable D4.4 is to validate the generation, and practical applicability of High Value New Resources (HVNR) obtained from the integrated treatment of urban, industrial, and organic waste streams within the SYMSITES framework. This includes demonstrating how waste can be efficiently transformed into valuable products aligned with circular economy principles.

More specifically, the deliverable aims to assess the technical performance and feasibility of different valorisation pathways, such as biogas and hydrogen production, recovery of platform molecules, production of biofertilizers, and generation of reclaimed water, using advanced technologies including AnMBR systems, pyrolysis, fermentation, and membrane processes.

Another key objective is to evaluate the quality and compliance of the recovered streams with current European and national regulations, particularly for applications in agriculture, industry, and urban environments. This includes verifying parameters such as heavy metals in sludge, microbiological safety, and water quality standards for reuse.

The deliverable also focuses on validating the performance of these resources in real operational environments, moving beyond laboratory-scale testing to pre-industrial and industrial conditions. This is demonstrated through applications such as textile dyeing processes, agricultural irrigation, and other industrial uses, ensuring that the recovered resources can effectively replace conventional raw materials without compromising performance.

In addition, D4.4 aims to identify the main technical, operational, and regulatory challenges associated with the upscaling and implementation of these solutions, including process stability, energy consumption, and potential barriers to market uptake.

Overall, the deliverable seeks to provide a comprehensive validation of the SYMSITES concept, confirming its potential to support resource efficiency, reduce environmental impact, and enable industrial symbiosis through the recovery and reuse of valuable resources from waste streams.



4 SPANISH ECOSITE

4.1. Description of the Ecosite

The main objective of the Spanish Ecosite is to treat industrial and urban wastewater (WW) from the municipality of Alcoy together with the urban organic solid waste from the town Muro de Alcoy to produce green hydrogen, high-quality reclaimed water and added-value products, through an anaerobic membrane bioreactor (AnMBR) and tertiary treatment. The three sources of waste in the Spanish Ecosite are:

- Urban organic solid waste generated and collected in the municipality of Muro de Alcoy.
- Urban and industrial WW from the municipal Alcoy WWTP
- Non-recyclable solid waste produced in the textile industry

The Spanish Ecosite allows five streams of new added-value products to be obtained as output (See Figure 1):

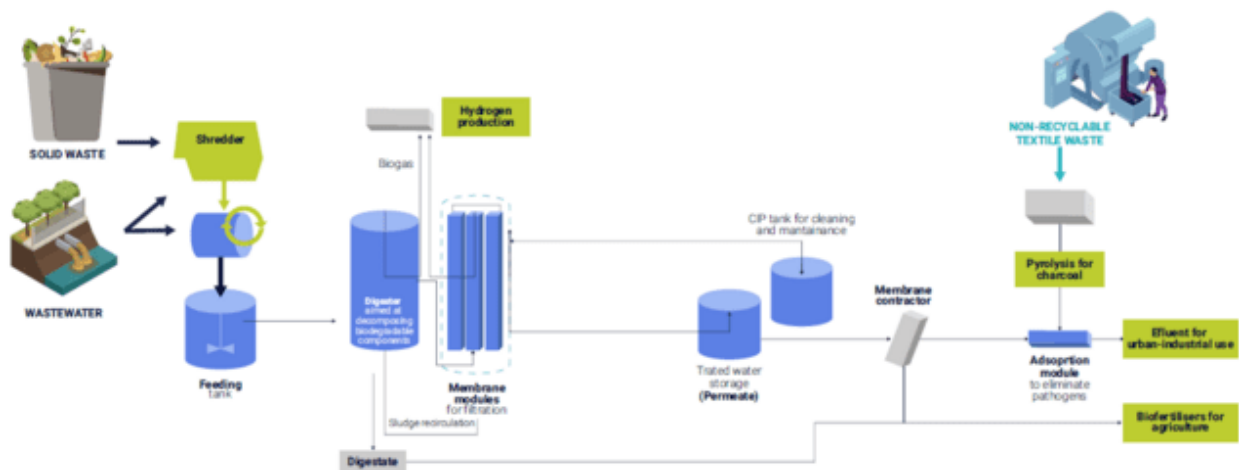


Figure 1. Spanish Ecosite: sources of waste, treatment processes and new added-value products

- Green hydrogen: CH_4 from AnMBR is reformed into H_2
- Digested sludge: tested for application as fertilizer
- Treated water: tested in industrial processes to replace all or part of the water employed conventionally
- Platform molecules: Benzoic or acetic acid produced from the liquid fraction of the pyrolysis treatment
- Charcoal: from pyrolysis of textile waste and transfer paper from the printing process stamps

4.2. Description of the work (results obtained)

In this point, all the work carried out, and the results obtained by the partners of the Spanish Ecosite are explained.

4.2.1. Biogas balance and hydrogen obtention from CH₄

This deliverable section presents the assessment of the biogas potentially producible within the SYMSITES process, including the estimated methane generation and the corresponding energy recovery potential. The analysis provides an overview of the expected biogas yields and their relevance in the context of Alcoy

For the baseline scenario, we started from the current operational data of the Alcoy WWTP together with data on the potential collection of municipal organic waste (OFMSW) from Alcoy, Muro de Alcoy, and Cocentaina.

Table 1. Potential amount of wastewater and OFMSW treated at the WWTP considering the municipalities of Muro de Alcoy, Alcoy and Cocentaina as well as the flow arriving to the Alcoy WWTP

Wastewater		
Flow treated	m ³ /day	13,485
Installed Power	kW	1,600
Sludge evacuated	kg	4,686,942
Dry matter	kg	930,844
OFMSW		
Alcoy	t/month	60
Muro de Alcoy	t/month	11.19
Cocentaina	t/month	9.13
Total	t/month	80.32

Using this data, we estimated the potential COD load available for anaerobic digestion and subsequently calculated the potential methane production that could be obtained daily from both wastewater and organic municipal waste streams.

Table 2. Biogas production potential

	Feed stream (m ³ /day; t/day)	COD (kg/m ³)	COD conversion (%)	m ³ CH ₄ /m ³ residual stream	m ³ CH ₄ /day
WW	13,485	0.704	70	0.17248	2,325.89
OFMSW	2,67	38.7	70	9.4815	25.38

Overall, the combined methane production potential for the baseline scenario is estimated at 2,351.3 m³ CH₄ /day, corresponding to an energy potential of approximately 23.5 MWh/day. These calculations provide an initial estimate of the renewable energy recovery potential associated with the integrated treatment of wastewater and municipal organic waste in the study area.

Considering that a digester can produce around 1600 m³/day (≈10.5 MWh/day), the adoption of the SYMSITES technology would mean a significant improvement in the auto sufficiency of the WWTP.

The significant increase in the estimated biogas production potential further highlights the importance of exploring advanced valorisation pathways, such as the conversion of biogas into renewable hydrogen. The obtention of this compound has been carried out by ITQ-CSIC.



In order to upgrade side streams, different routes to improve and reuse the different streams generated by the different wastes' treatments (AnMBR, AC, Pyrolysis) have been developed to produce high value new resources (HVNR). The biogas stream has been pre-treated and reformed for hydrogen production using protonic membrane reformer (PMR). This technology allows a single-step production, separation, and compression of H_2 from biogas/methane via the steam methane reforming (SMR) reaction while simultaneously producing a ready-to-capture CO_2 stream.

- **Development of protonic cells**

During task 2.1.1, protonic half-cells with planar geometry were successfully achieved at different scales, up to 25 cm^2 (see Figure 2), employing a new experimental procedure, sequential tape-casting. The characterization of these samples by XRD confirmed a single perovskite phase related to the BZCY532 electrolyte, where no secondary phases were detected. SEM images showed good attachment between the electrode support and the BCZY532 electrolyte. Besides, the electrode support showed that the mixture of BZCY532:NiO (60:40) was homogeneously distributed.

Stability measurements under H_2S atmospheres by XRD showed new peaks suggesting the formation of BaS. However, detailed analysis of the samples using SEM and EDS demonstrate structural homogeneity although sulphur had penetrated the structure.

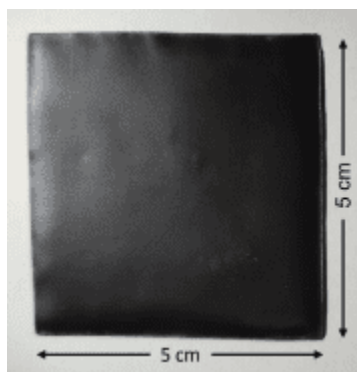


Figure 2. 25 cm^2 protonic half-cell.

Regarding the electrochemical characterization, (1,3 cm f) manufactured cells were evaluated by EIS measurements, it was concluded that the co-laminated production of protonic cells was successful, resulting in leak-tight and high-performance cells.

Now in Deliverable 4.4, electrochemical characterization of $5 \times 5\text{ cm}$ cells has been evaluated in pumping mode and the performance of these cells for H_2 production by steam methane reforming has been evaluated.

- **Electrochemical characterization of $5 \times 5\text{ cm}$ planar cells: pumping mode**

One sample of the co-laminated cell, $5 \times 5\text{ cm}$, was electrochemically characterized in pumping mode by I-V curves and the quantification of the faradaic efficiency was evaluated. A NiO/BZCY721 electrode was screen-printed onto the electrolyte.

Figure 3. a) shows the I-V curve obtained for the $5 \times 5\text{ cm}$ cell at $800\text{ }^\circ\text{C}$, using 500 mL/min of flow rate and an atmosphere containing 50% H_2 in Ar, humidified in H_2O (3 %). At 1V, 0.7 A/cm^2 is obtained. Figure 3. b) shows that the faradaic efficiency is 100% up to $0.5\text{ A}\cdot\text{cm}^2$ and the extracted H_2 flow reaches values higher than $60\text{ mL}\cdot\text{min}^{-1}$.



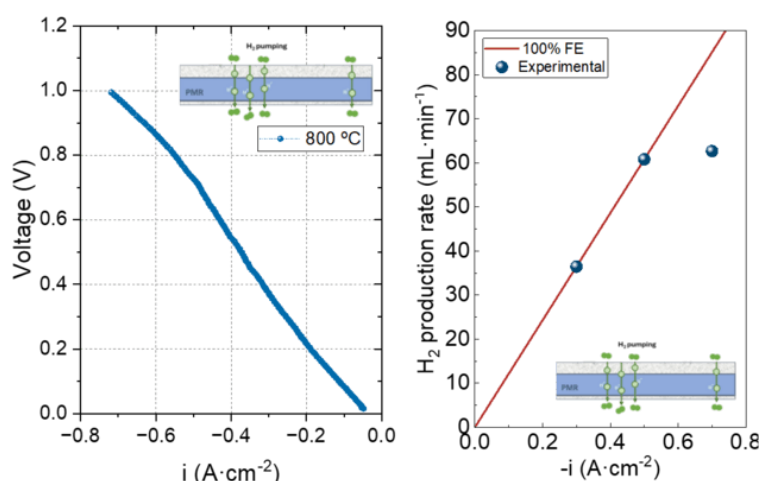


Figure 3. a) i -V curves and b) H₂ production rate vs current density.

- **PMR application for H₂ production**

The 5x5 cm cell performance was evaluated in the reaction of steam methane reforming (SMR). The reaction was carried out at 800 °C and different current densities, using 15 ml/min CH₄, 85 ml/min Ar, and 37.5 ml/min H₂O. Figure 4 shows that the conversion of CH₄ is almost quantitative, and the selectivity towards CO₂ increases by extracting H₂.

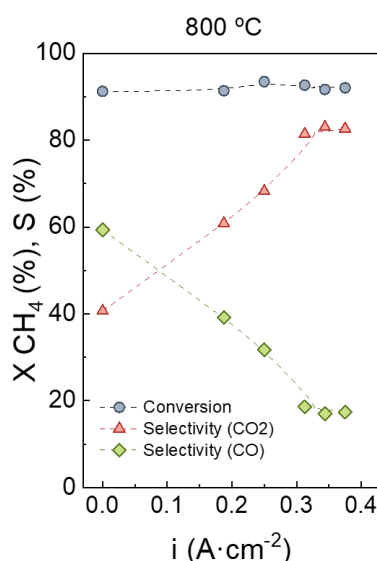


Figure 4. CH₄ conversion and selectivity towards CO₂ and CO (%).

4.2.2. Anaerobic sludge for application as biofertilizer

FACSA and FOVASA carried out an assessment of the regulatory framework governing the use of sewage sludge as fertilizers, considering both the applicable legislation and the physicochemical characteristics of the sludge. In addition, a preliminary study was conducted to estimate the amount of sludge with potential for agricultural application in the Alcoy area, including the identification of the critical heavy metals limiting its reuse potential.

Besides biogas, AD produces two main outputs: biogas, a renewable energy carrier, and digestate, a nutrient-rich residual stream. In WWTP, this digestate generally derives from the anaerobic stabilization of primary and/or secondary sewage sludge. In the case of SYMSITES, it comes from the joint digestion of the OFMSW and wastewater (urban and industrial). From a circular economy perspective, anaerobically digested sludge is seen as a potential biofertilizer, particularly because it contains organic matter, nitrogen, phosphorus, potassium and micronutrients that can partly substitute mineral fertilizers. In Spain, the relevance of this approach is reinforced by the national Biogas Roadmap, which identifies anaerobic digestion as a mature technology capable of integrating renewable energy production, organic waste management and circular economy strategies. The roadmap explicitly includes agricultural residues, agri-food residues, municipal biowaste and sewage sludge among relevant feedstocks, and sets a minimum target of 10.41 TWh/year of biogas production by 2030. The following parameters are of importance when determining the agronomic value of anaerobic sludge:

Table 3. Main parameters and relevance of each of them to determine the agronomic value of anaerobic sludge

Parameter	Relevance
Total solids / dry matter	Determines whether the material behaves as liquid, semi-solid or solid fertilizer
Organic matter / volatile solids	Contributes to soil carbon, structure and microbial activity, although anaerobic digestion reduces the most biodegradable fraction.
Total nitrogen	Main fertilizing parameter; part is organic N and part is mineral N.
Ammonium nitrogen, $\text{NH}_4^+\text{-N}$	Highly plant-available but also responsible for ammonia volatilization risk.
Phosphorus, P	Important for crop nutrition; may accumulate in soil if doses are based only on nitrogen
Potassium, K	Relevant especially for crops with high K demand.
pH	Digestate is usually neutral to alkaline, affecting nutrient availability and ammonia losses
Electrical conductivity	Indicates salinity risk, especially in repeated applications or sensitive crops.
C/N ratio	Usually lower than raw sludge; affects N mineralization and soil organic matter dynamics.
Heavy metals	Critical safety parameter, especially Cd, Cu, Ni, Pb, Zn, Hg and Cr.
Pathogens	Must be controlled through digestion, hygienization, storage or post-treatment.
Organic micropollutants	Pharmaceuticals, PFAS, pesticides, surfactants, microplastics and other emerging contaminants are increasingly relevant.
Stability / residual biogas potential	Indicates whether the material is sufficiently stabilized.
Phytotoxicity / germination index	Useful to assess potential negative effects on crops.



Compared with untreated sludge, anaerobic digestion stabilizes organic matter, reduces odour, lowers pathogen loads and converts part of the organic nitrogen into ammonium, making nitrogen more immediately available to crops. However, this same ammonium-rich nature increases the risk of NH_3 volatilization, nitrate leaching after nitrification, and crop toxicity if application is poorly managed. Recent reviews highlight digestate's potential to replace part of synthetic fertilizers but also stress that its performance depends strongly on feedstock, digestion conditions, storage, post-treatment and application method (Martín-Sanz, et al., 2025; Hagos, et al., 2026).

In practice, digestate may be used as:

- Whole digestate, applied directly to soil.
- Liquid fraction, rich in ammonium nitrogen and potassium, suitable for fertigation or surface/injection application.
- Solid fraction, richer in organic matter and phosphorus, often used as soil amendment or further composted. Recovered nutrient products, such as ammonium salts, struvite or precipitated phosphate salts.
- Composted or dried digestate, which improves handling, stability and marketability.

The use of anaerobically digested sludge as biofertilizer can provide several benefits:

- Reduces dependence on mineral fertilizers.
- Recovers nitrogen, phosphorus and organic carbon.
- Improves soil organic matter and structure.
- Supports circular economy in wastewater treatment.
- Reduces sludge disposal by landfill or incineration.
- Complements renewable energy production from biogas.
- Can reduce greenhouse gas emissions when replacing synthetic fertilizers and avoiding uncontrolled sludge degradation.

The main concerns are:

- Heavy metal accumulation in soil.
- Pathogens if hygienization is insufficient.
- Ammonia emissions during storage and field application.
- Nitrate leaching if application exceeds crop demand.
- Phosphorus accumulation in soils.
- Salinity and phytotoxicity, especially in sensitive crops.
- Odour and social acceptance issues.
- Emerging contaminants, including pharmaceuticals, PFAS, microplastics and antibiotic resistance genes.

EU legislation has historically focused mainly on heavy metals, while recent scientific and policy discussions increasingly call for broader monitoring of organic pollutants and environmental risks. At EU level, agricultural use of sewage sludge is primarily regulated by the Sewage Sludge Directive 86/278/EEC. Its purpose is to protect humans, animals, plants, soils and water by controlling the use of sewage sludge in agriculture, especially through limits on heavy metals in sludge and soils. It also regulates sludge and soil sampling, analysis, record keeping, quantities used, treatment type and application sites. For fertilising products placed on the EU market, Regulation (EU) 2019/1009 establishes product function categories and component material categories. Digestates are included



under specific component material categories (CMC): CMC 4: fresh crop digestate and CMC 5: digestate other than fresh crop digestate. However, CMC 5 explicitly excludes digestate produced from sewage sludge, industrial sludge or dredging sludge as eligible input materials for EU fertilising products. **This means that anaerobically digested sewage sludge may be used under national waste/sludge rules, but it does not automatically qualify as a CE-marked EU fertilising product under CMC 5.** In Spain, the agricultural use of sewage sludge is governed mainly by RD 1310/1990, **RD1051/2022** and the Orden AAA/1072/2013, which transpose the EU Sewage Sludge Directive and regulates the use of treated sewage sludge in agriculture. Furthermore, autonomous regions can promote their own legislation as depicted in next Table.

Table 4. Regulatory heterogeneity for sludge application in the agricultural field

AUTONOMUS REGION	TREATMENT	NORMATIVE
Andalucía	Sanitation biologic/chemical/thermal +	Orden 6 August 2018
Aragón	RD 1310/1990	RD 53/2019
Asturias	RD 1310/1990	National RD
Cantabria	RD 1310/1990	National RD
Castilla y León	Fermentation reduction	Functional sanitation
Castilla la Mancha	RD 1310/1990	No additional legislation
Cataluña	Anaerobic digestion	No additional legislation
Ceuta y Melilla	RD 1310/1990	National RD
Comunidad Valenciana	RD 1310/1990	Orden 22/2017; National RD
Extremadura	RD 1310/1990	National RD
Galicia	Biological, chemical, thermal, digestate or compost. Direct application banned	RD 125/2012
Islas Baleares	RD 1310/1990	National RD
Islas Canarias	RD 1310/1990	National RD
La Rioja	RD 1310/1990	National RD
Madrid	RD 1310/1990	RD 193/1998
Navarra	Composting, anaerobic digestion, storage > 30 days	Orden 359/2010
País Vasco	Advanced sanitation	RD 453/2013; Pathogen control
Murcia	RD 1310/1990	National RD

Current legislation not only establishes limit values for contaminants but also includes specific use restrictions to protect food safety and public health. For example, sludge cannot be applied to soils used for horticultural or fruit crops during their growing period, with the only exception being fruit trees. Moreover, this restriction is extended over a broader timeframe for certain crops: in the case of horticultural or fruit crops whose edible parts are typically in direct contact with the soil and are consumed fresh, the use of sludge is prohibited from ten months prior to the start of harvest until the end of the harvesting period.

The following section presents a detailed characterization of two types of sludge: on the one hand, a conventional sludge derived from a typical activated sludge wastewater treatment process, and on



the other hand, the sludge obtained from the pilot plant developed within SYMSITES. The results of this characterization are then benchmarked against the current regulatory framework, including European and national legislation governing the use of sewage sludge and derived materials in agriculture. This comparative assessment allows for **i) the identification of any exceedances of legal thresholds** or guideline values, thereby determining whether either sludge type presents potential limitations, as well as highlighting any additional treatment or management requirements needed to ensure compliance and environmental safety **ii) the use of the sludge as soil amendment**.

Table 5. Characterization and comparison of a conventional sludge and an anaerobic sludge obtained after SYMSITES treatment

Parameter	Units	SYMSITES	Conventional Sludge	Threshold value RD1051/2022
C/N Ratio		7	5,2	-
Total organic matter	% dm	77	72	>25%
Total CaO	mg/Kg (dm) CaO	46644	35509	-
Total MgO	mg/Kg (dm) MgO	7378	6759	-
NH₄	mg/Kg (dm) NH ₄	1,5	2,4	-
K₂O	mg/Kg (dm) K ₂ O	4071	7211	-
P₂O₅	mg/Kg (dm) P ₂ O ₅	16558	74836	-
Cu	mg/kg dm	228	221	1000
Zn	mg/kg dm	494	2300	2500
Cd	mg/kg dm	<2	<2	10
Cr	mg/kg dm	264	143	1000
Hg	mg/kg dm	0,4	0,7	10
Ni	mg/kg dm	45	146	300
Pb	mg/kg dm	95	49	750
Fe	mg/kg dm	33375	23587	-

From an agronomic perspective, both sludges exhibit a low C/N ratio (7 for SYMSITES and 5.2 for conventional sludge), which indicates a relatively stabilized organic matter and a tendency for rapid nitrogen mineralization once applied to soil. The total organic matter content is high in both cases (77% and 72%, respectively), clearly exceeding the minimum requirement of 25%, confirming their suitability as organic amendments capable of improving soil structure and fertility.

Regarding macronutrients, both materials contain significant amounts of calcium and magnesium oxides, with slightly higher values in the SYMSITES sludge. However, the most relevant differences appear in nutrient composition: the conventional sludge shows a remarkably higher phosphorus content (P₂O₅), while the SYMSITES sludge presents lower but still considerable levels. Potassium (K₂O) is higher in the conventional sludge, whereas ammonium nitrogen (NH₄⁺) remains relatively low in both cases, suggesting limited immediate nitrogen availability but reduced risk of ammonia volatilization.

In terms of heavy metals, both sludges comply comfortably with the legal thresholds, indicating no immediate restrictions for agricultural use from a regulatory standpoint. Elements such as Cu, Cd,



Cr, Hg, Ni and Pb are well below the maximum allowable limits in both cases. However, zinc (Zn) deserves particular attention: while still below the legal threshold (2500 mg/kg dm), the conventional sludge reaches relatively high levels (2300 mg/kg dm), approaching the regulatory limit and potentially requiring closer monitoring in long-term applications to avoid soil accumulation.

Finally, elements such as iron (Fe) are present in high concentrations in both sludges, which is typical for wastewater-derived materials and generally not limiting for agricultural use.

To evaluate the suitability of the SYMSITES sludge for agricultural application under the limits established by RD 1051/2022, a calculation was performed to determine the maximum allowable application rate (kg of dry sludge per hectare and year) based on heavy metal content. This approach ensures that the annual input of each regulated metal (Cu, Zn, Cd, Cr, Hg, Ni and Pb) does not exceed the legal thresholds expressed in g/ha·year. The calculation is based on a direct relationship between the concentration of each metal in the sludge (mg/kg dry matter) and its corresponding regulatory limit.

Table 6. Maximum sludge application taking into account the heavy metal concentration in the sludge

Metal	SYMSITES mg/kg dm	Limit RD g/ha·year	Max. kg dry sludge/ha·year
Cu	228	2400	10,526 kg
Zn	494	6000	12,146 kg
Cd	<2	150	>75,000 kg
Cr	264	2400	9,091 kg
Hg	0.4	150	375,000 kg
Ni	45	600	13,333 kg
Pb	95	1500	15,789 kg

By applying this method, a maximum permissible sludge dose is obtained for each metal, and the most restrictive value (*i.e.*, the lowest allowable dose) determines the overall application limit. **In this case, chromium (Cr) acts as the limiting factor, resulting in a maximum application rate of approximately 9.1 t of dry sludge per hectare and year. This ensures compliance with all heavy metal limits simultaneously**

Finally, the following section presents the physicochemical characterization of composts produced by different companies and agricultural cooperatives. These data are included for comparison purposes with compost values from literature.

Table 7. Physico-chemical characterization of two commercial compost¹

Parameter	Units	Compost 1	Compost 2	SYMSITES
Total organic matter	% dm	40	39	72
Total MgO	mg/Kg (dm) MgO	55,000	14,000	7,378
K ₂ O	mg/Kg (dm) K ₂ O	15,000	11,000	4,071

¹ Rupa-Huánuco, R. Caracterización y calidad de los compost producidos y comercializados en Rupa Rupa-Huánuco (Characterization and quality of compost produced and marketed in).



Parameter	Units	Compost 1	Compost 2	SYMSITES
P ₂ O ₅	mg/Kg (dm) P ₂ O ₅	15,000	10,000	16,558
Cu	mg/kg dm	42	42	228
Zn	mg/kg dm	780	70	494
Fe	mg/kg dm	3,029	3,500	33,375

- Balance applied to the generation and use of the anaerobic sludge

To know the total amount of sludge potentially generated in one year, we analysed the three municipalities (Alcoy, Cocentaina, and Muro de Alcoy) considering their area, population, agricultural surface, and main crop types. The agricultural activity in these municipalities is mainly based on olive groves, cereals, and other tree crops such as apple and almond trees so we assumed the surface of these soils to perform the calculations as soil to be amended.

Table 8. Main characteristics of the municipalities studied within the SYMSITES project

	Alcoy	Cocentaina	Muro de Alcoy
Size (km ²)	129,9	52,94	30,24
Inhabitants	61613	11486	9372
Surface dedicated to agriculture (Ha)	5955	1569	1202,5
Kind of agriculture	olive groves, cereals and other trees (such as apples or almonds)		

Based on the amount of organic matter expected to be treated in the WWTP process, sludge production was estimated using a yield coefficient of 0.1 kg SSV/kg COD removed. The calculations indicate a sludge generation of approximately 977 kg TS per day, equivalent to 356,796 kg TS per year (considering 13,485 m³/day of WW and 2,67 t/day of OFMSW). The total agricultural land available in the three municipalities is 8,726.5 hectares. When distributing the generated sludge over this agricultural area, the resulting application rate is approximately 40.89 kg /ha·year.

Considering the maximum allowable sludge application rate of 9.1 t TS/ha·year established by the chromium limit, only around 40 hectares of agricultural land would be required to safely dispose of the total annual sludge production. Therefore, the available agricultural surface in the studied municipalities is largely sufficient to absorb all the sludge generated within the system, leaving a significant surplus of land available for additional applications if needed. These results are below the limit previously established for land application. Therefore, all the sludge generated could be safely reused within these municipalities as soil amendment. In addition, there would still be surplus capacity available, meaning that part of the sludge could also be applied to agricultural land in nearby areas.

It should be stressed that a hygienization step would be required prior to any agricultural application depending on the final destination. Current regulatory frameworks might include microbiological quality criteria upon final destination, typically requiring the analysis of indicators such as *Escherichia coli* and *Salmonella spp.* to ensure sanitary safety. In SYMSITES, the sludge has been produced under ambient temperature conditions, which means that the process does not guarantee the



inactivation or removal of pathogenic microorganisms. As a result, the elimination of these microorganisms has not been ensured, and therefore additional treatment (such as thermal drying, pasteurization or other hygienization methods) would be necessary to comply with microbiological standards and minimize potential health risks.

In summary, both sludges potentially meet current regulatory requirements and present good potential for soil amendment. Nevertheless, the conventional sludge shows higher phosphorus and Zn contents, which may influence application strategies, particularly in soils already rich in phosphorus or under repeated application scenarios.

4.2.3. Reuse of treated wastewater

RECLAIMED WATER SUITABILITY

Taking into account the results from Deliverable 4.3, a stream of reclaimed water was obtained for both microfiltration and ultrafiltration membranes. This water has different potential uses according to the RD 1085/2024. FACSA and FOVASA have assessed the suitability of this reclaimed water to be used for the following applications:

- Urban use and potential applications in an I-U symbiosis environment
- Agricultural use and potential applications in an I-U symbiosis environment
- Industrial use and potential applications in an I-U symbiosis environment

• Urban use and potential applications in an I-U symbiosis environment

Below is shown a table with the required water quality standards and their corresponding applications in various urban use contexts (**Table 9**). The table outlines specific quality criteria and examples of how water can be utilized. Each quality class is linked to specific urban applications based on safety and environmental requirements.

Table 9. Different quality requirements for urban use of water and classification of the water according to these characteristics

Class of Quality	<i>E. coli</i> (CFU/100 mL)	Turbidity (UNT)	SS (mg/L)	Intestinal Nematodes (eggs/10L)	Bacteriophages (PFU/100mL)
A+	Absence	5	10	1	100 (when there is a risk of aerosolization)
- Private garden irrigation. - Discharge from sanitary devices.					
A.	10	5	10	1	100 (when there is a risk of aerosolization)
- Publicly accessible ornamental ponds and circulating water.					
B.	100		35	-	-
- Street cleaning. - Irrigation of green urban areas (parks and similar). - Firefighting systems. - Industrial vehicle washing.					
C.	1,000		35	-	-
- Non-accessible ornamental ponds and circulating water.					



Based on the provided data, the treated water obtained with UF membranes is preliminarily classified as **Quality A** according to the official standards for urban use. This classification is justified by the low levels of *E. coli* (3 CFU/100 mL), which meet the threshold of up to 10 CFU/100 mL but do not achieve the "absence" required for U. A+ classification. Additionally, the total bacteriophages concentration (8 PFU/100 mL) is well below the maximum allowed limit of 100 PFU/100 mL for A. It is important to note that other parameters required by law, such as *Legionella* spp., or intestinal nematodes have been analyzed resulting in absence of these pathogens.

- **Agricultural use and potential applications in an I-U symbiosis environment**

The following table presents the water quality standards required for various agricultural applications. Similarly, it highlights the specific criteria needed for safe and effective use of water in agricultural practices. Depending on the water quality requirements for reclaimed water used in agricultural irrigation, Quality A permits all irrigation methods for crops consumed raw, where the edible part is in direct contact with reclaimed water, as well as tuber crops. Quality B also allows all irrigation methods but is limited to crops consumed raw where the edible part grows above the soil and is not in direct contact with reclaimed water. Quality C applies to methods that prevent direct contact with the edible part of crops, including non-food crops, transformed food crops, and crops used to feed livestock. It also covers woody crops with edible fruits and ornamental plants grown in nurseries or greenhouses without direct water contact. Finally, quality D permits all irrigation methods but is restricted to non-food crops, energy production, and seed production.

Table 10. Different quality requirements for agricultural use of water and classification of the water according to these characteristics

Class	<i>E. coli</i> (CFU/100 mL)	Turbidity (NTU)	SS (mg/L)	BOD ₅ (mg/L)	Intestinal Nematodes (eggs/L)	<i>Legionella</i> spp. (CFU/L)	<i>T. saginata</i> and <i>T. solium</i> (eggs/L)
A	10	5	10	10	1 for irrigation of pastures or forage	< 1,000 when aerosolization risk exists	–
B	100	–	35	25	1 for irrigation of pastures or forage	< 1,000 when aerosolization risk exists	1 for pastures of meat-producing animals
C	1,000	–	35	25	1 for irrigation of pastures or forage	< 1,000 when aerosolization risk exists	1 for pastures of meat-producing animals
D	10,000	–	35	25	1 for irrigation of pastures or forage	< 1,000 when aerosolization risk exists	–

The treatment results, when compared to the regulatory standards for agricultural reuse, indicate that the treated water generally meets the requirements for **Quality B**. In particular, microbiological quality is high, with *E. coli* concentrations around 3 CFU/100 mL, well below the threshold for **Quality A** (10 CFU/100 mL). Additionally, other key parameters such as BOD₅ ($\approx$ 5 mg/L, compared to a maximum limit of 10 mg/L for Quality A), as well as the absence of intestinal nematodes, *Legionella* spp., and *Taenia saginata* and *Taenia solium* eggs, fully comply with the most stringent criteria.

However, parameters related to suspended solids and turbidity show greater variability. The measured values of TSS (8.5 ± 6.2 mg/L) and turbidity (16.1 ± 26.7 NTU) in the permeate indicate that, while average TSS concentrations are close to the Quality A limit (10 mg/L), turbidity exceeds



the corresponding threshold (5 NTU). This discrepancy is mainly driven by a limited number of high values, which increase the overall average and variability.

These results suggest that, although the system currently achieves **Quality B**, it has the **potential to reach Quality A** under optimized and stable operating conditions. In particular, improving process control to minimize occasional performance deviations could ensure consistent compliance with the stricter limits for TSS and turbidity, while the rest of the parameters already meet the highest quality standards.

- Industrial use and potential applications in an I-U symbiosis environment**

Table 11. Different quality requirements for industrial use of water, classification of the water according to these characteristics, and potential uses.

Class	<i>E. coli</i> (CFU/100 mL)	Turbidity (NTU)	SS (mg/L)	<i>Legionella</i> spp. (CFU/L)
A+	0	5	10	Comply with RD 487/2022
Cooling towers and evaporative condensers in the water supplied to the tower				
C	1,000	–	35	Comply with RD 487/2022

Process water: Water that comes into direct contact at any point with raw materials, materials, intermediate products, or final products in an industrial process.

Cleaning water: Water intended for cleaning equipment, objects, materials, pipes, and surfaces in industrial areas, except in food processing facilities.

Since SYMSITES treatment process does not completely eliminate *E. coli*, the treated water would be classified as **Quality C** based on the standards outlined in **Table 11**, where some uses and applications are depicted. There is a special case considered within the law regarding food processing companies. In these cases, stricter water quality requirements apply, particularly concerning suspended solids (SS), which must be below 10 mg/L and turbidity (below 5 NTU). Given that the treated water in SYMSITES does not meet this threshold of turbidity, it would be restricted in its use within food processing facilities. Specifically, the treated water could be used during food manufacturing processes for purposes such as cooling, or the production of steam or hot water in closed-loop systems, which do not come into contact with food.

- Environmental use and potential applications in an I-U symbiosis environment**

Table 12. Different quality requirements for environmental use of water, classification of the water according to these characteristics, and potential uses.

Environmental Destination	<i>E. coli</i> (UFC/100 mL)	Turbidity (NTU)	SS (mg/L)	Intestinal Nematodes (eggs/10L)	Bacteriophages (UFP/100mL)
Quality A+	Absence	5	10	1	100 (only when the aquifer is for supply purposes)
Artificial recharge of aquifers by direct injection.					
Quality C	1,000	-	35	-	-



Environmental Destination	<i>E. coli</i> (UFC/100 mL)	Turbidity (NTU)	SS (mg/L)	Intestinal Nematodes (eggs/10L)	Bacteriophages (UFP/100mL)
Artificial recharge of aquifers by localized percolation through the ground.					
Quality to be defined	Case-by-case study	-	-	-	-
Contribution of water resources to wetlands and other aquatic ecosystems.					

The treatment carried out in the project does not guarantee the absence of *E. coli* or such a low concentration of turbidity as required for higher quality standards (A+). Therefore, the resulting water quality could initially be classified as **Quality C**, given the limitations in achieving stricter thresholds for these parameters.

In summary, the treatment carried out in this project aligns with the quality standards required for various applications, including urban, environmental, and industrial uses. The process demonstrates its ability to meet these standards effectively. However, depending on the specific intended use, it will be necessary to evaluate whether the treatment can consistently provide reclaimed water of the required quality. This adaptability ensures that the process remains versatile and capable of addressing diverse water quality needs.

Regarding the quality of the water obtained through the microfiltration membrane process for the different evaluated reuse applications, the results indicate that the system performance is strongly dependent on the operating conditions applied. Under engine optimized conditions, a clear reduction in turbidity and suspended solids is observed, allowing the treated water to approach the quality requirements for the intended reuse applications. However, under less favorable operating regimes, certain parameters such as TSS may remain above the target thresholds, indicating the need for further optimization to ensure consistent compliance. In terms of microbiological quality, additional assessment is required, particularly for fecal indicators such as *E. coli*. Due to the larger pore size of microfiltration membranes, a lower removal efficiency is obtained in comparison with UF systems. Therefore, further investigation is needed to better characterize this aspect and define potential complementary strategies if required.

Evaluating a real scenario for water reuse in the Alcoy region

A potential implementation scenario for the Alcoy WWTP considers the installation of an alternative treatment line based on the SYMSITES technology developed within the project. In this scenario, 50 % of the influent flow entering the WWTP would be diverted and treated through the SYMSITES line, while the remaining flow would continue to be processed through the existing conventional treatment system.

Considering an average influent flow rate of 13,485 m³/day, the proposed scenario would therefore treat approximately 6,742.5 m³/day of wastewater. The reclaimed water produced through this treatment line could subsequently be reused for urban applications, such as street washing, irrigation of green areas, or other municipal services managed by FOVASA.

The following table presents the updated water balance and operational assumptions associated with this scenario.



Table 13. Water Balance and Operational Assumptions for the 50% Influent Flow Treatment Scenario Using the SYMSITES Line at Alcoy WWTP

Concept	Unit	Assumed Value	Comment
WWTP influent flow rate	m ³ /day	13485	Flow inlet to WWTP
Percentage of reclaimed water production	%	50	Initial assumption
Reclaimed water available	m ³ /day	6742,5	
Population of Alcoy	inhabitants	61500	
Municipal area	km ²	129.9	
Considered urban area	km ²	8	Urban área (Alcoy + Muro de Alcoy)
Percentage of urban road surface	%	25	Streets, sidewalks, squares and paved areas
Total road surface area	m ²	2000000	
Street washing water demand	L/m ² ·operation	1.5	
Average washing frequency	operations/week	2	Two washing operations per week
Weekly washing demand	m ³ /week	6000	
Average daily demand	m ³ /day	857	Q _{week} / 7
Daily water balance	m ³ /day	5885	Water available for additional uses
Percentage of reclaimed water used	%	13	

The proposed street washing scenario would utilize approximately 13% of the reclaimed water produced. Therefore, a significant volume of regenerated water would still remain available for additional reuse applications. In particular, the remaining reclaimed water could potentially be allocated for agricultural irrigation purposes, including its use as Class B reclaimed water for irrigation.

Irrigation reuse scenario

In this scenario, after allocating approximately 13% of this reclaimed water to street washing, the remaining volume is considered potentially available for irrigation. Under Quality B, all irrigation methods may be used, but application is limited to crops consumed raw where the edible part grows above the soil and is not in direct contact with reclaimed water. This makes the scenario suitable for tree crops, provided that reclaimed water does not come into direct contact with the edible part.



Table 14. Water Balance and Operational Assumptions for the use of regenerated water for agricultural applications

Concept	Unit	Value	Comment
Reclaimed water available for irrigation	m ³ /day	5885.0	
Annual reclaimed water available for irrigation	m ³ /year	2141081	
Alcoy agricultural area	ha	5955	
Cocentaina agricultural area	ha	1569	
Muro de Alcoy agricultural area	ha	1202	
Total agricultural area	ha	8726	Sum of the three municipalities
Assumed irrigation demand	m ³ /ha-year	4500	Typical annual demand for Mediterranean tree crops
Irrigable area with available reclaimed water	ha	475,8	
Agricultural area devoted for woody crops	ha	1,000	
Share of total agricultural area covered	%	47.58	

The results indicate that approximately 2.14 million m³/year of reclaimed water could be made available for agricultural irrigation after accounting for the volume allocated to street washing. Under the assumed irrigation demand, this would allow the irrigation of approximately 476 hectares of agricultural land using Quality B reclaimed water. The total agricultural area considered across Alcoy, Cocentaina and Muro de Alcoy exceeds 8,700 hectares, from which around 1,000 are devoted to woody crops, meaning that half of this area could be supplied under the current treatment scenario. Given the larger extension of agricultural land in the study area, future implementation strategies could consider reducing or delimiting the irrigation scheme to a smaller area or to a single municipality to improve feasibility and operational management.

Alternatively, the treatment capacity of the SYMSITES line at the Alcoy WWTP could be increased beyond the current 50% influent flow scenario, thereby increasing the volume of reclaimed water available for irrigation reuse and other applications such as industrial uses, further developed in the next section. However, any future increase in water reuse applications should be carefully balanced to ensure that environmental flows and water masses within the public hydraulic domain are not adversely affected, while also preventing negative impacts on downstream users and existing water uses and applications.

INDUSTRIAL REUSE OF THE RECLAIMED WATER - VALIDATION FOR DYEING PROCESSES IN THE TEXTILE INDUSTRY

AITEX in collaboration with JOVER have validated the reuse of the treated wastewater in the Spanish Ecosite for dyeing processes at pre-industrial and industrial scale.



The use of the high value new resources (HVNR), in this case the treated wastewater of the Spanish EcoSite in Alcoy, was validated in WP2 for its use in dyeing processes at a lab scale. It is necessary to validate the use of this treated WW in relevant operational environments to generate the symbiosis and replace the virgin water for industrial processes. For this purpose, the work of AITEX in WP4 corresponds to the scaleup of the dyeing processes with WW at both semi-industrial and industrial scale.

Below, the results obtained on the characterization of the dyed samples for pre-industrial and industrial scaleup are explained in detail.

The description of the ingredients, material, equipment and dyeing processes carried out are included in the **ANNEX I_AITEX_(a) Description of equipment and materials**. The images of the dyed fabrics as well as the graphs including the values measured of L^* , a^* and b^* for all the replicates are included in **ANNEX I_AITEX_(b) Fabrics dyed and Boxplots with L^* , a^* and b^* results**.

All dyed samples were characterized by colorimetry and washing fastness to evaluate the quality of the dyeing process. The technologies used are detailed in the deliverable 1.2., and the results are detailed below.

- **Colourimetry**

FABRIC DYEING

- PRE-INDUSTRIAL SCALEUP

Unitary blue over polyester Fabric

The following table shows the average of the 5 measurements taken from the colourimeter on each sample. It is easy to observe that there is almost no difference among the three samples carried out in each experiment.

Table 15. L^* , a^* and b^* coordinates average measurements for blue polyester fabric.

% Process water	Sample	L^* average	a^* average	b^* average
100%	S1	47,41	-1,49	-36,20
	S2	47,21	-1,49	-36,23
	S3	47,95	-1,92	-35,95
85%	S1	46,99	-1,73	-36,03
	S2	46,56	-1,95	-37,39
	S3	46,62	-2,12	-37,40
75%	S1	45,57	-1,83	-37,35
	S2	45,92	-2,07	-36,79
	S3	46,60	-2,27	-36,72

Finally, the difference in colour between the reference (100% process water) and the samples dyed with 15% and 25% of WW from the Pilot is obtained by calculating the parameter ΔE^* .



Table 16. Calculation of the ΔE^* for 15% and 25% wastewater compared to the reference. Blue polyester fabric.

Experiment	% Process water	L*average	a*average	b*average	$\Delta L^*_{100\%}$	$\Delta a^*_{100\%}$	$\Delta b^*_{100\%}$	ΔE^*
Blue-Polyester fabric PP water	100%	47,52	-1,63	-36,13	NA			
	85%	46,72	-1,93	-36,94	-0,802	-0,30	-0,81	1,18
	75%	46,03	-2,06	-36,95	-1,491	-0,43	-0,82	1,76

According to the evaluation scale described previously, the dyeing of blue polyester using 15% and 25% of WW from the Pilot Plant shows a very small deviation, barely visible (ΔE^*_{1-2}) from the reference (100% process water).

Trichromy over polyester fabric

The following table shows the average of the 5 measurements taken from the colourimeter on each sample. It is easy to observe that there is almost no difference among the three samples carried out in each experiment.

Table 17. L*, a* and b* coordinates average measurements for trichromy polyester fabric.

% Process water	Sample	L*average	a*average	b*average
100%	S1	51,74	-18,21	26,76
	S2	51,40	-18,60	26,93
	S3	50,94	-18,49	26,49
85%	S1	51,55	-18,38	27,06
	S2	50,76	-18,79	26,67
	S3	51,30	-18,57	26,98
75%	S1	51,99	-18,67	26,86
	S2	52,35	-18,65	26,91
	S3	51,47	-18,47	26,35

Finally, the difference in colour between the reference (100% process water) and the samples dyed with 15% and 25% of WW from the Pilot is obtained by calculating the parameter ΔE^* .

Table 18. Calculation of the ΔE^* for 15% and 25% wastewater compared to the reference. Trichromy polyester fabric.

Experiment	% Process water	L*average	a*average	b*average	$\Delta L^*_{100\%}$	$\Delta a^*_{100\%}$	$\Delta b^*_{100\%}$	ΔE^*
Trichromy Polyester 2 Tint control PP water	100%	51,36	-18,43	26,73	NA			
	85%	51,20	-18,58	26,91	-0,16	-0,15	0,18	0,28
	75%	51,94	-18,60	26,71	0,57	-0,17	-0,02	0,60

According to the evaluation scale described previously, the dyeing of trichromy polyester using 15% and 25% of WW from the Pilot Plant shows a very small deviation, barely visible ($\Delta E^* < 1$) from the reference (100% process water).

Unitary dye over cotton fabric



The following table shows the average of the 5 measurements taken from the colourimeter on each sample. It is easy to observe that there is almost no difference among the three samples carried out in each experiment.

Table 19. L^* , a^* and b^* coordinates average measurements for unitary cotton fabric.

% Process water	Sample	L^* average	a^* average	b^* average
100%	S1	41,58	-6,34	-24,45
	S2	41,88	-6,51	-24,84
	S3	42,74	-6,79	-24,77
85%	S1	40,57	-6,68	-25,49
	S2	41,36	-6,96	-25,41
	S3	41,09	-6,83	-24,92
75%	S1	40,79	-6,54	-24,85
	S2	41,54	-6,79	-25,13
	S3	40,33	-6,44	-24,95

Finally, the difference in colour between the reference (100% process water) and the samples dyed with 15% and 25% of WW from the Pilot is obtained by calculating the parameter ΔE^* .

Table 20. Calculation of the ΔE^* for 15% and 25% wastewater compared to the reference. Unitary cotton fabric.

Experiment	% Process water	L^* average	a^* average	b^* average	$\Delta L^*_{100\%}$	$\Delta a^*_{100\%}$	$\Delta b^*_{100\%}$	ΔE^*
Blue-Cotton Tint control 2 PP water	100%	42,06	-6,55	-24,69	NA			
	85%	41,01	-6,82	-25,27	-1,057	-0,28	-0,58	1,24
	75%	40,89	-6,59	-24,98	-1,177	-0,04	-0,29	1,21

Despite the problem with the white stains, the blue obtained is quite similar for the samples and the reference since ΔE^* is <2 (difference from the reference barely appreciated).

Trichromy over cotton fabric

The following table shows the average of the 5 measurements taken from the colourimeter on each sample. It is easy to observe that there is almost no difference among the three samples carried out in each experiment.

Table 21. L^* , a^* and b^* coordinates average measurements for trichromy over cotton fabric.

% Process water	Sample	L^* average	a^* average	b^* average
100%	S1	57,32	-5,00	27,23
	S2	57,52	-5,06	26,95
	S3	57,32	-5,13	27,08
85%	S1	56,87	-5,03	25,83
	S2	56,01	-5,54	25,36
	S3	56,01	-5,61	25,00
75%	S1	55,55	-5,51	26,02



% Process water	Sample	L*average	a*average	b*average
	S2	56,56	-5,05	26,57
	S3	56,60	-5,04	26,48

Finally, the difference in colour between the reference (100% process water) and the samples dyed with 15% and 25% of WW from the Pilot is obtained by calculating the parameter ΔE^* .

Table 22. Calculation of the ΔE^* for 15% and 25% WW compared to the reference. Trichromy over cotton fabric.

Experiment	% Process water	L*average	a*average	b*average	$\Delta L^*_{100\%}$	$\Delta a^*_{100\%}$	$\Delta b^*_{100\%}$	ΔE^*
Trichromy Cotton 2 Tint control PP water	100%	57,39	-5,06	27,09	NA			
	85%	56,30	-5,39	25,40	-1,09	-0,33	-1,69	2,04
	75%	56,24	-5,20	26,36	-1,15	-0,13	-0,73	1,37

According to the evaluation scale described previously, the dyeing of trichromy cotton using 15% and 25% of WW from the Pilot Plant shows a small deviation barely visible (ΔE^*_{1-2}) from the reference (100% process water).

- INDUSTRIAL SCALEUP

Unitary yellow over cotton fabric

The following table shows the average of the 5 measurements taken from the colourimeter on each sample.

Table 23. L*, a* and b* coordinates average measurements for unitary over cotton fabric.

% Process water	Sample	L*average	a*average	b*average
100	S1	78,99	-2,01	52,59
	S2	78,85	-1,91	52,81
	S3	78,90	-1,88	52,69
85	S1	78,24	-1,29	52,75
	S2	78,04	-1,32	52,75
	S3	78,24	-1,27	52,89
75	S1	77,96	-0,68	53,52
	S2	78,17	-0,77	53,54
	S3	78,05	-0,82	53,22

Finally, the difference in colour between the reference (100% process water) and the samples dyed with 15% and 25% of WW from the Pilot is obtained by calculating the parameter ΔE^* .

Table 24. Calculation of the ΔE^* for 15% and 25% WW compared to the reference. unitary over cotton fabric.

Experiment	% Process water	L*average	a*average	b*average	$\Delta L^*_{100\%}$	$\Delta a^*_{100\%}$	$\Delta b^*_{100\%}$	ΔE^*
Unitary cotton	100%	78,91	-1,93	52,70	NA			



Washing Machine, PP	85%	78,12	-1,14	52,98	-0,79	0,79	0,28	1,16
	75%	78,06	-0,76	53,43	-0,85	1,18	0,73	1,63

According to the evaluation scale described previously, the dyeing of unitary cotton using 15% and 25% of WW from the Pilot Plant shows a small deviation barely visible (ΔE^*1-2) from the reference (100% process water).

Trichromy over cotton fabric

The following table shows the average of the 5 measurements taken from the colourimeter on each sample. It is easy to observe that there is almost no difference among the three samples carried out in each experiment.

Table 25. L^* , a^* and b^* coordinates average measurements for trichromy over cotton fabric.

% Process water	Sample	L^* average	a^* average	b^* average
100%	S1	55,64	1,65	4,52
	S2	55,73	1,66	4,77
	S3	55,54	1,70	4,88
85%	S1	55,13	0,47	2,62
	S2	54,86	0,55	3,16
	S3	54,84	0,40	3,16
75%	S1	55,66	2,39	2,71
	S2	55,33	2,38	2,62
	S2	55,59	2,46	2,96

Finally, the difference in colour between the reference (100% process water) and the samples dyed with 15% and 25% of WW from the Pilot is obtained by calculating the parameter ΔE^* .

Table 26. Calculation of the ΔE^* for 15% and 25% WW compared to the reference. Trichromy over cotton fabric.

Experiment	% Process water	L^* average	a^* average	b^* average	$\Delta L^*_{100\%}$	$\Delta a^*_{100\%}$	$\Delta b^*_{100\%}$	ΔE^*
Trichromy, Grey cotton Washing Machine, PP	100%	55,64	1,67	4,72	NA			
	85%	54,94	0,47	2,98	-0,69	-1,20	-1,74	2,22
	75%	55,53	2,41	2,76	-0,11	0,74	-1,96	2,10

According to the evaluation scale described previously, the dyeing of trichromy cotton in washing machine using 15% and 25% of WW from the Pilot Plant shows a small deviation ($\Delta E^*>2$) from the reference (100% process water).

Unitary blue over acrylic fabric

The following table shows the average of the 5 measurements taken from the colourimeter on each sample. It is easy to observe that there is almost no difference among the three samples carried out in each experiment.



Table 27. L^* , a^* and b^* coordinates average measurements for unitary over acrylic fabric.

% Process water	Sample	L^* average	a^* average	b^* average
100%	S1	48,52	-6,15	-33,89
	S2	48,93	-6,31	-33,89
	S3	48,46	-6,17	-34,09
85%	S1	48,88	-7,2	-33,76
	S2	48,87	-7,2	-33,60
	S3	48,82	-7,07	-33,68
75%	S1	48,19	-6,72	-33,94
	S2	47,94	-6,63	-33,92
	S3	48,38	-6,76	-33,73

Finally, the difference in colour between the reference (100% process water) and the samples dyed with 15% and 25% of WW from the Pilot is obtained by calculating the parameter ΔE^* .

Table 28. Calculation of the ΔE^* for 15% and 25% WW compared to the reference. Unitary over acrylic fabric.

Experiment	% Process water	L^* average	a^* average	b^* average	$\Delta L^*_{100\%}$	$\Delta a^*_{100\%}$	$\Delta b^*_{100\%}$	ΔE^*
Unitary blue, acrylic Washing Machine, PP	100%	48,64	-6,21	-33,96	NA			
	85%	48,86	-7,16	-33,68	0,22	-0,94	0,28	1,01
	75%	48,17	-6,71	-33,87	-0,47	-0,49	0,09	0,69

According to the evaluation scale described previously, the dyeing of unitary acrylic in washing machine using 15% and 25% of WW from the Pilot Plant shows that the difference from the reference is barely appreciated ΔE^* is <2 .

Trichromy over acrylic fabric

The following table shows the average of the 5 measurements taken from the colourimeter on each sample. It is easy to observe that there is almost no difference among the three samples carried out in each experiment.

Table 29. L^* , a^* and b^* coordinates average measurements for trichromy over acrylic fabric.

% Process water	Sample	L^* average	a^* average	b^* average
100%	S1	44,73	10,70	-0,47
	S2	42,74	10,94	-0,54
	S3	45,00	10,69	-0,44
85%	S1	45,77	10,38	-0,09
	S2	46,16	10,26	-0,13
	S3	45,58	10,51	0,01
75%	S1	45,81	10,66	0,11
	S2	45,56	10,63	0,09
	S3	45,32	10,65	0,10



Finally, the difference in colour between the reference (100% process water) and the samples dyed with 15% and 25% of WW from the Pilot is obtained by calculating the parameter ΔE^* .

Table 30. Calculation of the ΔE^* for 15% and 25% WW compared to the reference. Trichromy over acrylic fabric.

Experiment	% Process water	L*average	a*average	b*average	$\Delta L^*_{100\%}$	$\Delta a^*_{100\%}$	$\Delta b^*_{100\%}$	ΔE^*
Trichromy, Acrylic Washing Machine, PP	100%	44,15	10,78	-0,48	NA			
	85%	45,84	10,38	-0,07	1,68	-0,39	0,41	1,78
	75%	45,56	10,65	0,10	1,41	-0,13	0,58	1,53

According to the evaluation scale described previously, the dyeing of trichromy acrylic in washing machine using 15% and 25% of WW from the Pilot Plant shows that the difference from the reference is barely appreciated ΔE^* is <2 .

Unitary blue over Wool fabric

The following table shows the average of the 5 measurements taken from the colourimeter on each sample. It is easy to observe that there is a little difference among the three samples carried out in each experiment (specially the one dyed with 75% Pilot Plant water).

Table 31. L*, a* and b* coordinates average measurements for unitary over wool fabric.

% Process water	Sample	L*average	a*average	b*average
100%	S1	55,11	-11,15	-15,36
	S2	55,07	-11,25	-15,49
	S3	55,32	-11,12	-15,68
85%	S1	55,03	-12,17	-13,67
	S2	55,01	-12,16	-13,89
	S3	54,94	-12,14	-13,81
75%	S1	57,13	-12,13	-11,06
	S2	56,79	-11,99	-10,88
	S3	56,92	-11,85	-10,87

Finally, the difference in colour between the reference (100% process water) and the samples dyed with 15% and 25% of WW from the Pilot is obtained by calculating the parameter ΔE^* .

Table 32. Calculation of the ΔE^* for 15% and 25% WW compared to the reference. Unitary over wool fabric.

Experiment	% Process water	L*average	a*average	b*average	$\Delta L^*_{100\%}$	$\Delta a^*_{100\%}$	$\Delta b^*_{100\%}$	ΔE^*
Unitary blue, Wool Washing Machine, PP	100%	55,17	-11,17	-15,51	NA			
	85%	54,99	-12,16	-13,79	-0,18	-0,99	1,72	1,99
	75%	56,95	-11,99	-10,94	1,78	-0,82	4,57	4,98



According to the evaluation scale described previously, the dyeing of unitary wool in washing machine using 15% of WW from the Pilot Plant shows that the difference from the reference is barely appreciated ΔE^* is <2 .

However, the value obtained for 25% of WW shows a much higher difference from the reference compared, with visible colour differences appreciated ΔE^* is >2 .

Trichromy over Wool fabric

The following table shows the average of the 5 measurements taken from the colourimeter on each sample. It is easy to observe that the difference among the three samples carried out in each experiment is very small.

Table 33. L^* , a^* and b^* coordinates average measurements for Trichromy over Wool fabric.

% Process water	Sample	L^* average	a^* average	b^* average
100%	S1	51,65	7,24	11,12
	S2	52,04	7,28	10,90
	S3	51,69	7,23	11,09
85%	S1	51,71	7,39	11,78
	S2	51,17	7,42	11,69
	S3	51,63	7,43	11,68
75%	S1	49,40	8,39	10,85
	S2	49,17	8,23	10,69
	S3	49,68	8,42	10,78

Finally, the difference in colour between the reference (100% process water) and the samples dyed with 15% and 25% of WW from the Pilot is obtained by calculating the parameter ΔE^* .

Table 34. Calculation of the ΔE^* for 15% and 25% WW compared to the reference. Trichromy over wool fabric.

Experiment	% Process water	L^* average	a^* average	b^* average	$\Delta L^*_{100\%}$	$\Delta a^*_{100\%}$	$\Delta b^*_{100\%}$	ΔE^*
Trichromy Wool Washing Machine PP water	100%	51,80	7,25	11,04	NA			
	85%	51,50	7,42	11,72	-0,30	0,16	0,67	0,75
	75%	49,42	8,35	10,77	-2,38	1,09	-0,27	2,63

According to the evaluation scale described previously, the dyeing of trichromy wool in washing machine using 15% of WW from the Pilot Plant shows that the difference from the reference is barely appreciated ΔE^* is <2 . However, when 25% of WW is used, there is a small deviation can be appreciated ΔE^* >2 .

Due to wool's fibre chemical structure (amino groups) and the dyeing mechanism of acid dyes (electrostatically interaction between the protonated amino groups of wool under acidic conditions with the sulfonic groups of the acid colorant), the use of wastewater for dyeing these fibres makes them more susceptible to interactions between their functional groups and the quality parameters from the water treated by the pilot plant.



The higher conductivity, the presence of organic matter and suspended solids may interfere with the electrostatic interactions between the fibre and the colorant, resulting higher ΔE^* values compared to other nature fibres. Nevertheless, the dyeing tests results were uniform and showed no defects.

THREAD DYEING

Unitary red over polyester thread

The following table shows the average of the 5 measurements taken from the colourimeter on each sample. It is easy to observe that the difference among the three samples carried out in each experiment is very small.

Table 35. L^* , a^* and b^* coordinates average measurements for Unitary over polyester yarn.

% Process water	Sample	L^* average	a^* average	b^* average
100%	S1	33,71	53,63	14,77
	S2	35,41	55,05	15,28
	S3	35,38	55,15	15,2
85%	S1	33,84	55,07	17,72
	S2	34,18	54,81	16,63
	S3	34,7	54,74	16,77
75%	S1	33,56	53,76	17,45
	S2	35,29	54,58	15,99
	S3	35,26	54,73	16,17

Finally, the difference in colour between the reference (100% process water) and the samples dyed with 15% and 25% of WW from the Pilot is obtained by calculating the parameter ΔE^* .

Table 36. Calculation of the ΔE^* for 15% and 25% WW compared to the reference. Unitary over polyester yarn.

Experiment	% Process water	L^* average	a^* average	b^* average	$\Delta L^*_{100\%}$	$\Delta a^*_{100\%}$	$\Delta b^*_{100\%}$	ΔE^*
Unitary Polyester Yarn, Bellini, PP water	100%	34,83	54,61	15,08	NA			
	85%	34,70	54,36	16,54	-0,13	-0,25	1,45	1,48
	75%	34,24	54,87	17,04	-0,59	0,26	1,96	2,06

According to the evaluation scale described previously, the dyeing of unitary polyester yarn using 15% and 25% of WW from the Pilot Plant shows a small deviation barely visible (ΔE^*1-2) from the reference (100% process water).

Trichromy military green over polyester thread

The following table shows the average of the 5 measurements taken from the colourimeter on each sample. It is easy to observe that the difference among the three samples carried out in each experiment is very small.

Table 37. L^* , a^* and b^* coordinates average measurements for Trichromy over polyester yarn.

% Process water	Sample	L^* average	a^* average	b^* average
-----------------	--------	---------------	---------------	---------------

100%	S1	33,36	1,19	13,2
	S2	34,37	1,37	14,04
	S3	32,87	1,33	13,45
85%	S1	34,31	0,6	12,72
	S2	33,61	0,86	13,73
	S3	33,39	0,88	13,26
75%	S1	33,29	0,71	13,08
	S2	33,23	0,83	13,17
	S3	33,05	0,73	12,88

Finally, the difference in colour between the reference (100% process water) and the samples dyed with 15% and 25% of WW from the Pilot is obtained by calculating the parameter ΔE^* .

Table 38. Calculation of the ΔE^* for 15% and 25% WW compared to the reference. Trichromy over polyester yarn.

Experiment	% Process water	L*average	a*average	b*average	$\Delta L^*_{100\%}$	$\Delta a^*_{100\%}$	$\Delta b^*_{100\%}$	ΔE^*
Trichromy Polyester Yarn, Bellini, PP water	100%	33,53	1,30	13,56	NA			
	85%	33,77	0,78	13,24	0,24	-0,52	-0,33	0,66
	75%	33,19	0,76	13,04	-0,34	-0,54	-0,52	0,82

According to the evaluation scale described previously, the dyeing of trichromy polyester yarn in the yarn industrial machine using 15% and 25% of WW from the Pilot Plant shows that the difference from the reference is barely appreciated ΔE^* is <1.

- **Washing fastness**

In the following Tables are shown the results of washing fastness obtained in the different dyeings carried out.

Table 39. Results washing fastness semi-industrial scaleup: Unitary dye over polyester fabric

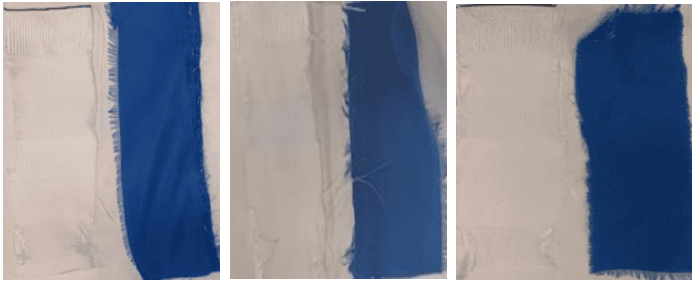
Washing Fastness: Blue dye over Polyester fabric (semi-industrial)				
Discharge Fabric	VALUE	100% Process water	85% Process water	75% Process water
Triacetate	5			
Cotton	5			
Polyamide	4/5			
Polyester	5			
Polyacrylic	5			
Viscose	5			
Degradation Fabric	VALUE			
Polyester Fabric	5			

Table 40. Results washing fastness semi-industrial scaleup: trichromy over polyester fabric

Washing Fastness: Green trichromy dye over Polyester fabric (semi-industrial)				
Discharge Fabric	VALUE	100% Process water	85% Process water	75% Process water
Triacetate	5			
Cotton	5			
Polyamide	4/5			
Polyester	5			
Polyacrylic	5			
Viscose	5			
Degradation Fabric	VALUE			
Polyester Fabric	5			

Table 41. Results washing fastness semi-industrial scaleup: unitary dye over cotton fabric

Washing Fastness: Blue dye over Cotton fabric (semi-industrial)				
Discharge Fabric	VALUE	100% Process water	85% Process water	75% Process water
Triacetate	5			
Cotton	5			
Polyamide	4/5			
Polyester	5			
Polyacrylic	5			
Viscose	5			
Degradation Fabric	VALUE			
Polyester Fabric	5			

Table 42. Results washing fastness semi-industrial scaleup: trichromy over cotton fabric

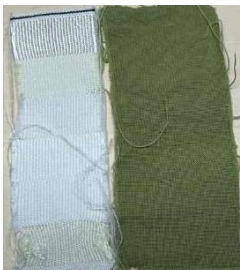
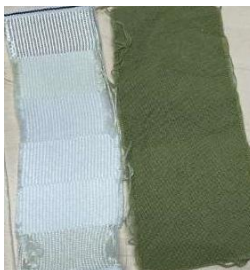
Washing Fastness: Green trichromy dye over Cotton fabric (semi-industrial)				
Discharge Fabric	VALUE	100% Process water	85% Process water	75% Process water
Triacetate	5			
Cotton	5			
Polyamide	4/5			
Polyester	5			
Polyacrylic	5			
Viscose	5			
Degradation Fabric	VALUE			
Polyester Fabric	5			



Table 43. Results washing fastness industrial scaleup: unitary dye over acrylic fabric

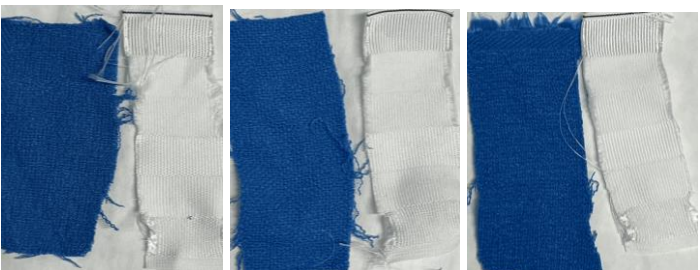
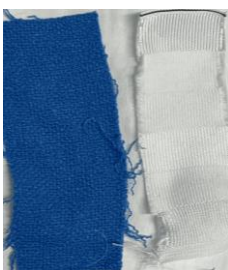
Washing Fastness: Blue dye over Acrylic fabric (Industrial)				
Discharge Fabric	VALUE	100% Process water	85% Process water	75% Process water
Triacetate	5			
Cotton	4/5			
Polyamide	5			
Polyester	5			
Polyacrylic	5			
Viscose	5			
Degradation Fabric	VALUE			
Acrylic Fabric	5			

Table 44. Results washing fastness industrial scaleup: trichromy dye over acrylic fabric

Washing Fastness: Trichromy over Acrylic fabric (Industrial)				
Discharge Fabric	VALUE	100% Process water	85% Process water	75% Process water
Triacetate	5			
Cotton	4/5			
Polyamide	5			
Polyester	5			
Polyacrylic	5			
Viscose	5			
Degradation Fabric	VALUE			
Acrylic Fabric	5			

Table 45. Results washing fastness industrial scaleup: unitary dye over Wool fabric

Washing Fastness: Blue dye over Wool fabric (Industrial)				
Discharge Fabric	VALUE	100% Process water	85% Process water	75% Process water
Triacetate	5			
Cotton	4/5			
Polyamide	3			
Polyester	5			
Polyacrylic	5			
Viscose	4/5			
Degradation Fabric	VALUE			
Wool Fabric	5			



Table 46. Results washing fastness industrial scaleup: trichromy dye over Wool fabric

Washing Fastness: Brown trichromy dye over Wool fabric (Industrial)				
Discharge Fabric	VALUE	100% Process water	85% Process water	75% Process water
Triacetate	5			
Cotton	5			
Polyamide	4/5			
Polyester	5			
Polyacrylic	5			
Viscose	5			
Degradation Fabric	VALUE			
Wool Fabric	5			

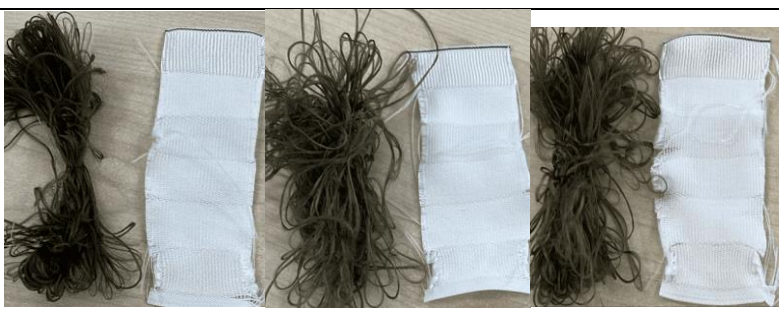
Table 47. Results washing fastness industrial scaleup: unitary dye over Polyester Thread

Washing Fastness: Red unitary dye over Polyester Thread (Industrial)				
Discharge Fabric	VALUE	100% Process water	85% Process water	75% Process water
Triacetate	5			
Cotton	5			
Polyamide	5			
Polyester	5			
Polyacrylic	5			
Viscose	5			
Degradation Fabric	VALUE			
Polyester Thread	5			

Table 48. Results washing fastness industrial scaleup: trichromy dye over Polyester Thread

Washing Fastness: Trichromy military green dye over Polyester Thread (Industrial)				
Discharge Fabric	VALUE	100% Process water	85% Process water	75% Process water
Triacetate	5			
Cotton	5			
Polyamide	5			
Polyester	5			
Polyacrylic	5			
Viscose	5			



Degradation Fabric	VALUE	
Polyester Thread	5	

As can be seen in the Tables from above, all the results obtained in the washing fastness test of the dyed samples showed no difference between the samples dyed using the different percentages of wastewater from the Pilot Plant and the samples dyed with process water (reference).

- **Conclusions**

After all the results obtained on the dyeing procedures carried out in semi-industrial and industrial scale of the different nature fabrics and thread, and the characterization results obtained in the dyed samples with wastewater from the Pilot Plant, it can be said that it is possible to obtain a quality dye with the use of 15% and 25% Pilot Plant water.

The use of wastewater treated from the Spanish Ecosite in JOVER's textiles production processes can lead to savings in water supply of around 7533 m³ per year, thereby reducing their costs for these resources.

POST-PROJECT INDUSTRIAL UPTAKE AND FOLLOW-UP ACTIONS (JOVER CASE STUDY)

As a direct outcome of the successful results obtained during the SYMSITES project in the validation of treated wastewater reuse for textile dyeing processes, additional follow-up activities have been initiated by industrial partners beyond the formal scope of the project.

In particular, the positive performance observed when using treated wastewater from the Spanish EcoSite Pilot Plant—demonstrating that the incorporation of 15–25% reclaimed water does not negatively affect process stability or final fabric quality—has encouraged Jover to further explore water reuse strategies within its own facilities.

Although these activities are not funded under the SYMSITES project, they are a direct consequence of the knowledge and confidence generated during its implementation.

To evaluate the feasibility of internal water reuse, Jover collected and analyzed samples from the effluent of its existing wastewater treatment plant. A comparison was carried out between the physicochemical parameters of the pilot plant water—successfully used during the project—and Jover's current effluent (**Table 49**), in order to assess its suitability for dyeing applications.

Table 49. Comparison between the physicochemical parameters of Pilot Plant water and Jover's treatment plant effluent

PHYSICOCHEMICAL CHARACTERIZATION OF WASTEWATER						
	pH	Conductivity (μS/cm)	SST(mg/L)	COD (mgO ₂ /L)	Turbidity (UT)	Color (PT-CO)
Pilot plant water	6,6	1969,3	2,3	137	31,4	61,7
Jover's treatment plant effluent	7,67	2706,78	79	859,18	-	-



The results indicate that key parameters such as conductivity, suspended solids (TSS), and COD are significantly higher in Jover's effluent than in the pilot plant water. These parameters are critical in textile dyeing processes, as they directly influence dye uptake, process reproducibility, and final product quality. Therefore, the current quality of Jover's treated wastewater does not yet meet the requirements for direct reuse in dyeing operations.

In response to these findings, Jover has initiated a technical study to upgrade its wastewater treatment system through the implementation of a membrane bioreactor (MBR). This technology combines biological treatment with membrane filtration, allowing for a more efficient removal of organic matter, suspended solids, and turbidity compared to conventional systems.

The implementation of the MBR is expected to significantly improve effluent quality, aligning it more closely with the characteristics of the pilot plant water validated within SYMSITES. This will enable the reuse of internally treated wastewater in dyeing processes, reducing freshwater consumption and supporting a more sustainable and circular water management strategy.

Overall, this case clearly demonstrates the strong exploitation potential of the SYMSITES' results, as well as their ability to trigger additional investments and innovation actions beyond the project's duration.

INDUSTRIAL REUSE OF THE RECLAIMED WATER - VALIDATION FOR REACTOR CLEANING IN THE COSMETIC INDUSTRY

In parallel with cosmetic product development, this work addressed the validation of reactor cleaning processes through the reuse of treated wastewater, contributing to circular economy strategies and more sustainable water management within the cosmetic industry.

In this context, cleaning and disinfection protocols combining alkaline and acidic treatments were evaluated using treated wastewater from two different sources. On the one hand, treated wastewater from the **Ecosite España pilot plant** was used, and on the other hand, treated wastewater generated in the internal treatment process of the cosmetic industry **Germaine**, a project partner (**GERM**), which actively collaborated in the development and validation of these trials.

The evaluation of both types of treated wastewater made it possible to compare their performance under real industrial cleaning conditions while maintaining the same operational and control criteria. The results obtained demonstrated that, when appropriate control and disinfection measures are applied, wastewater can be safely reused for the cleaning of cosmetic reactors without compromising microbiological safety or cleaning effectiveness.

Furthermore, the use of treated wastewater originating from the cosmetic industry (GERM) highlighted the potential for internal water reuse within the sector itself, reinforcing the feasibility of sustainable solutions adapted to real industrial environments.

Detailed physicochemical and microbiological results, as well as the corrective actions implemented during the validation process, are presented in **Annex IV (d)**.

- **Conclusions**

The work carried out highlights the multifunctional value of ectoine in cosmetic applications, particularly its moisturizing efficacy and its contribution to strengthening the skin barrier function. These effects were confirmed through externally conducted *in vivo* studies, in which relevant



improvements were observed in key skin parameters such as hydration, elasticity and firmness following the application of formulations containing this active ingredient.

Furthermore, the comparative studies performed demonstrated a statistically significant improvement in skin hydration for ectoine-containing formulations when compared with placebo products, reinforcing the suitability of ectoine as a functional ingredient for the development of cosmetic products aimed at skin care and protection.

In addition to the cosmetic efficacy results, the validation of reactor cleaning processes through the reuse of treated wastewater demonstrates the feasibility of implementing more sustainable practices within cosmetic manufacturing. The findings show that, under appropriate control and disinfection conditions, treated wastewater can be safely reused without compromising microbiological safety or cleaning effectiveness, contributing to a more efficient and responsible use of water resources.

The detailed experimental procedures, datasets, statistical analyses and validation evidence supporting these conclusions are provided in the annexes referenced throughout this deliverable.

4.2.4. Platform molecules from the pyrolysis system

BENZOIC ACID FROM THE PYROLYSIS SYSTEM

SYMSITES aims to transform non-recyclable wastes into valuable resources, thus improving waste valorisation within an Industrial-Urban Symbiosis framework. In this context, the work of GREENE focuses on the validation of platform molecules obtained from the pyrolysis system, with particular emphasis on the recovery, purification and application assessment of benzoic acid from textile-derived pyrolytic liquids generated in the Spain EcoSite. This approach is consistent with the project objective of converting complex waste streams into high-value resources with potential industrial use, although benzoic acid has recognised preservative uses in other industrial sectors as cosmetic and detergency industry, within SYMSITES the selected validation route was textile application in connection with the Spain Ecosite value chain.

Below, a summary of the results by Greene is shown. The **Annex II_Greene_Recovery, Purification and functionalization** collect all the experiments carried out and results obtained in detail.

- **Identification of the target platform molecule in textile-derived pyrolytic liquids**

The validation activities carried out by GREENE focused on the liquid fraction generated during the pyrolysis of non-recyclable textile waste in the Spanish EcoSite. Previous characterisation work showed that this fraction separates into an organic and an aqueous phase, the latter being particularly relevant for downstream chemical valorisation due to its content of oxygenated and aromatic compounds.

GC-MS analysis of the aqueous phase confirmed that benzoic acid and related benzoic derivatives are the dominant compounds present in this stream (see Figure 5). This analytical result was decisive for the definition of the validation strategy, as it demonstrated that the liquid fraction obtained from real textile pyrolysis conditions contains a clearly identifiable and potentially recoverable platform molecule. The selection of benzoic acid was therefore based on experimental evidence rather than on theoretical assumptions, ensuring that the validation route remained grounded in realistic process conditions.



Qualitative Report

File: C:\TurboMass\Instalacion.PRO\Data\Muestra 1281.raw
Acquired: 13-Dec-23 01:00:11 PM
Description: Con acetato de etilo
GC/MS Method: GC: Aceites 45min_Manual.mth MS: Aceites 45min.EXP
Sample ID: D26-COND-793-S0052
Printed: 13-Dec-23 01:46 PM
Page 1 of 1
Vial Number: 10

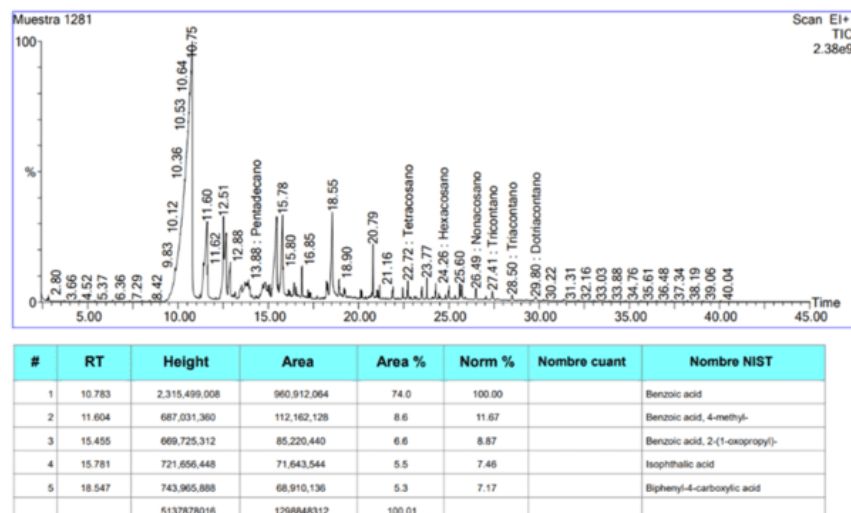


Figure 5. GC-MS results for the aqueous phase obtained from textile waste pyrolysis in the Spain EcoSite, showing benzoic acid and benzoic derivatives as the main compounds of interest.

In addition to its analytical relevance, benzoic acid was considered suitable for validation due to its compatibility with conventional separation and purification techniques and its potential functional use in sectors directly linked to the textile value chain. This combination of chemical traceability and application relevance justified its selection as the target High Value New Resource within the framework of WP4.

- **Validation of the recovery route by liquid-liquid extraction**

The recovery of benzoic acid from the aqueous phase of the textile-derived pyrolytic liquid was validated using liquid-liquid extraction, building on the process developed in WP2. Among the solvents previously evaluated, dimethyl ether (DME) was selected due to its favourable extraction behaviour, low operating temperature and suitability for solvent recovery.

Experimental extraction tests carried out on real aqueous samples showed that a single extraction step achieved an average recovery of approximately 50% of the benzoic acid present. When a second extraction stage was applied using fresh solvent, the cumulative extraction efficiency increased to 75.2%. This result demonstrates that a relatively simple two-stage extraction cascade (see Figure 6) can significantly enhance recovery without increasing process complexity.

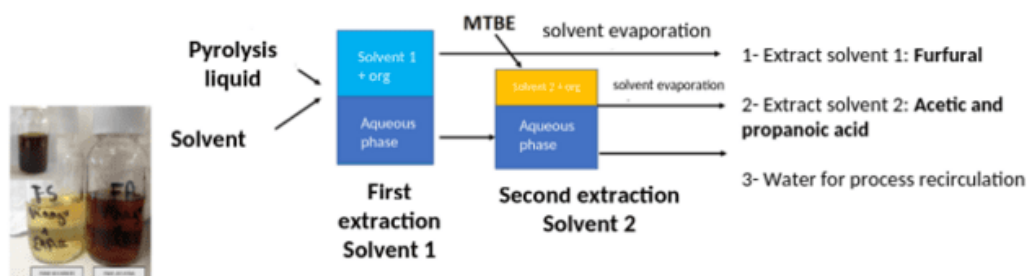


Figure 6. Liquid-liquid extraction selected as the most suitable route for benzoic acid recovery from the textile-derived aqueous phase.

Based on these results, the overall material conversion was estimated at 81% with respect to the aqueous phase generated during pyrolysis. From a process validation perspective, this finding is highly relevant, as it confirms that the aqueous fraction, often considered a low-value by-product, can act as a realistic source of a recoverable platform molecule. The extraction step therefore constitutes a technically feasible and reproducible route for benzoic acid recovery from textile-derived pyrolytic liquids.

- **Purification of the recovered benzoic acid**

Although solvent evaporation after extraction yielded a solid product, further purification was required to ensure suitability for formulation and application-oriented validation. Recrystallisation was selected as the purification method, given its effectiveness in improving the purity of organic solids when the target compound is already present at high concentration.

The initial solid obtained after extraction showed a purity of approximately 74%. After two successive recrystallisation stages (see Figure 7), the purity increased to 88%, as confirmed by chromatographic analysis. This improvement demonstrates that the recovered benzoic acid can be upgraded through a relatively simple downstream operation, bridging the gap between recovery and functional assessment.



Figure 7. Experimental process followed for benzoic acid recrystallisation.

From a validation standpoint, these results confirm that the recovery route is not limited to analytical isolation but can deliver a product with a quality level compatible with further formulation work. The



purification step therefore represents a key element in the overall platform molecule validation pathway.

- **Formulation development and antifungal performance in solution**

The functional validation of the recovered benzoic acid focused on its potential use as an antifungal and anti-odour agent for textile materials during storage. The first formulation approach involved the conversion of benzoic acid into sodium benzoate by neutralisation, in order to improve solubility and handling in aqueous systems.

However, antifungal tests performed with sodium benzoate solutions at pH 5.5 did not show inhibition of *Candida albicans* or *Aspergillus niger* (see Figure 8). While negative from a performance perspective, this result was informative, as it highlighted the importance of chemical speciation in determining antimicrobial activity. Subsequent analysis confirmed that the biologically active form is mainly the non-dissociated benzoic acid, which is only present in significant amounts under acidic conditions

Fotografía con el microscopio estereoscópico
Photography with the stereoscopic microscope

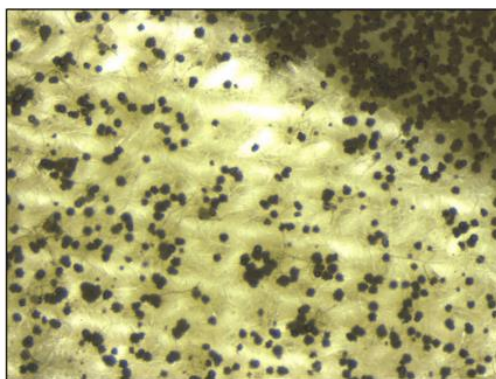


Figure 8. Fungal growth visual assessment

Based on this insight, a second formulation (AF_1) was developed using a water/propylene glycol mixture adjusted to acidic pH with citric acid. Under these conditions, antifungal screening according to AATCC Test Method 30-2017 showed complete inhibition of both target microorganisms in solution. This result confirmed that the recovered benzoic acid retains its intrinsic antifungal functionality when the formulation environment is appropriately controlled.

- **Transfer of antifungal functionality to textile substrates**

Following the positive results obtained in solution, the AF_1 formulation was applied to textile substrates using different application methods. Initial trials using foulard impregnation resulted in limited antifungal performance on cotton fabrics, even when process parameters were modified to increase pick-up. Although some improvement was observed, complete inhibition of fungal growth was not achieved under these conditions.

To enhance deposition, the application route was changed to spraying, allowing higher amounts of formulation to be retained on the textile surface (126% for polyester and 134% for cotton). This approach produced the most promising results of the application stage, with no growth of *Candida albicans* (Figure 9) observed on either cotton or polyester fabrics. In contrast, *Aspergillus niger* growth was not effectively suppressed (Figure 10), indicating that the antifungal effect is



microorganism-dependent and strongly influenced by the deposited amount and application conditions.

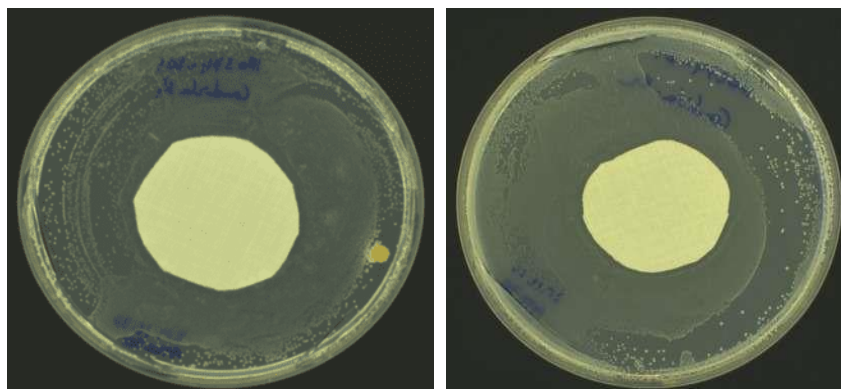


Figure 9. Results of the test against *Candida albicans* after spray application. Left polyester fabric and right cotton fabric

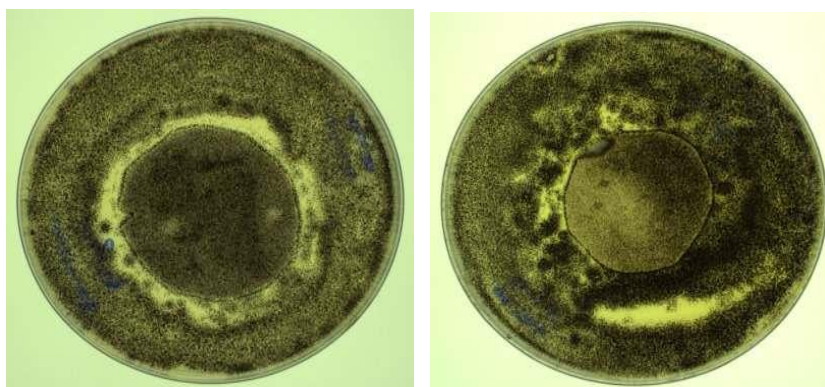


Figure 10. Results of the test for *Aspergillus niger*. Left on polyester fabric and right on cotton fabric

Conclusions

Overall, the results obtained by GREENE demonstrate a coherent and traceable validation pathway from molecule recovery to application-oriented assessment. The work confirms that benzoic acid can be recovered from textile-derived pyrolytic aqueous phases with good efficiency, purified to a suitable quality level and formulated to exhibit antifungal activity.

VALIDATION OF ECTOINE PROPERTIES

- **Introduction and Context**

Ectoine is a naturally derived extremolyte obtained from methane-rich biogas streams and is well known for its osmoprotective, moisturizing and skin barrier-stabilizing properties. At the molecular level, ectoine binds water molecules, forming protective hydration shells around proteins and lipids. At the skin level, this mechanism translates into improved barrier integrity and a reduced transepidermal water loss (TEWL).



Based on these properties, several ectoine-based cosmetic formulations were developed and evaluated within the project to assess their effects on skin hydration, barrier function, elasticity and firmness, as well as their applicability in cosmetic manufacturing and sustainable cleaning processes.

- **Development of Ectoine-Based Cosmetic Products (Summary)**

Within the framework of the project, ectoine-containing cosmetic formulations were developed at laboratory and semi-industrial scale through the collaboration of **AITEX** and the cosmetic industry partner **Germaine (GERM)**. The developments included a leave-on serum and a rinse-off facial cleansing gel, designed to evaluate the cosmetic performance and applicability of ectoine in different product formats.

The formulation work focused on ensuring physicochemical stability, appropriate rheological behavior, and compatibility of ectoine with other cosmetic ingredients, as well as on assessing the scalability of the formulations under conditions representative of industrial manufacturing.

Detailed information regarding formulation composition, stability studies, rheological characterization and scale-up procedures is provided in **Annex IV (a)**.

- **In Vivo Efficacy Assessment of the Ectoine Serum (External Studies)**

All *in vivo* efficacy studies involving human volunteers were carried out externally by specialized and accredited testing facilities and were not performed directly by the European project partners.

The externally conducted *in vivo* tests were performed using a multi-probe micro-analysis system on a panel of 21 volunteers. The following probes were used to evaluate skin performance parameters:

- **Corneometer® CM 825:** Surface hydration
- **Tewameter® TM 300:** Transepidermal water loss (TEWL)
- **Cutometer® dual MPA 580:** Skin elasticity and firmness

The efficacy of ectoine was validated by AITEX through externally conducted *in vivo* studies. The results obtained demonstrated improvements in skin hydration, elasticity and firmness after 28 days of application of the ectoine-containing serum compared to baseline values.

Subsequently, a comparative study was carried out in which the ectoine-containing serum was applied to one half of the face, while a placebo formulation was applied to the contralateral side. In this study, a statistically significant improvement in skin hydration was observed on the ectoine-treated side compared to the placebo, confirming the moisturizing efficacy of ectoine under comparative use conditions.

A complete description of the study design, statistical methodology and numerical results is provided in **Annex IV (b)**.

- **Anti-Irritation Performance of the Ectoine Facial Gel (External Study)**

An ectoine-containing facial cleansing gel was developed to evaluate the ability of this active ingredient to mitigate surfactant-induced skin irritation. The anti-irritation efficacy study was conducted externally using an *in vivo* protocol based on repeated washing and TEWL measurements.



As expected for rinse-off products, TEWL increased on both the ectoine-treated and placebo-treated skin areas. However, the ectoine-treated side showed a lower mean increase in TEWL compared to the placebo, indicating improved skin barrier resilience. Although the differences between treatments did not reach statistical significance, the observed trend suggests a protective effect of ectoine against surfactant-induced barrier disruption.

Detailed experimental data and statistical outputs are included in **Annex IV (c)**.



5 DANISH ECOSITE

5.1 Description of the Ecosite

The main objective of the Danish Ecosite is to investigate the prospects of biogas production for industrial use and filtered WW for agricultural irrigation. The WW of the Danish Ecosite comes from the Svaneke Brewery and the Nearby town of Svaneke. The ground food waste is added to increase the volume of biogas. The filtered WW is analysed to determine its applicability to agricultural irrigation at Frennegård in accordance with the EU regulation. Sludge from the test facility is used as fertilizer at Frennegård if the quality is suitable.

The advanced WW treatment pilot facility consists of multiple interrelated processes (see diagram in **Figure 11**):

- Pretreatment: grinder, agitators, dosing pumps and drum filter
- Bioreactor to produce biogas and sludge
- Membrane unit to produce filtered water

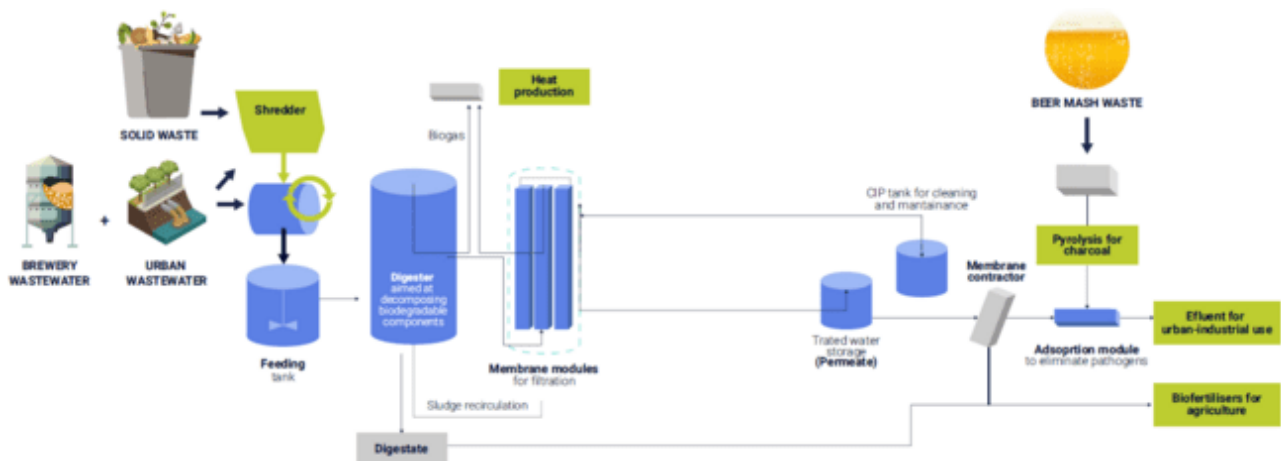


Figure 11. Danish Ecosite: sources of waste, treatment processes and new added-value products

As a result of, High-Value New Resources are available: treated wastewater for irrigation, digested sludge for fertiliser, biogas rich in methane and char from the Beer mash.

5.2. Description of the work (results obtained)

The Danish EcoSite, as already described in the previous section, investigated four different valorization routes, including **water for fertigation**, **digestate as biofertilizer**, **biogas** as renewable energy and **biochar**.

Produced **sludge or digestate** was characterized as part of **deliverable D.4.3** and compared against Danish regulation for heavy metals. In most periods it could be classified as sludge type A, which can be applied on land. PFAS were also detected, which may change the sludge classification if the legislation changes. If so, sludge would be classified as B and it would need to undergo mineralization process before its application in land.

Biogas quantity and composition was also presented in **D.4.3**, including the energy content it has. Before its application as energy source, biogas needs to be desulphurized (i.e., remove the H_2S) in order to protect the co-generation engine. After burning, both electricity and heat are generated.

Alternatively, biogas can be upgraded through physic-chemical processes that remove carbon dioxide, thereby producing biomethane (equivalent to natural gas) and carbon dioxide, which can be further valorized or captured. This second option is most complex and usually only suitable for relatively large biogas productivity.

In the following sections, **both fertigation and biochar** are presented in more detail, including the tests performed to assess the value of the recovered streams.

5.2.1 Treated wastewater for fertigation

• Methodology in fertigation trials for permeate reuse

Willow transpiration tests

The willow toxicity experiments were carried out as adapted from the ISO 8692. The test follows the principle that healthy trees transpire more water than sick trees. Cuttings of *Salix Viminalis* are grown hydroponically in sealed flasks, first in nutrient solution (mimicking standard fertilizers – **Figure 12**) and then in the solutions that are tested for.

From: ISO International Organization for Standardization (1997) Water quality - Fresh water algal growth test with *Scenedesmus subspicatus* and *Raphidocelis subcapitata*. ISO Standard 8692. International Organization for Standardization, Geneva, Switzerland

Modified ISO 8692 nutrient solution

Modified	ISO 8692	Final conc.	Molv.	Stock conc.	ml/l	Stock conc (Mol/l)
		mg/l	g/mol	$\mu\text{Mol/L}$	g/l	
STAM						
mod. A	NaNO ₃	240,00	84,99	2823,71	48,00	5 ml in 1 L
B	MgCl ₂ ·6H ₂ O	12,00	203,30	59,03	12,00	1 ml in 1 L
C	CaCl ₂ ·2H ₂ O	18,00	147,02	122,44	18,00	1 ml in 1 L
D	MgSO ₄ ·7H ₂ O	15,00	246,47	60,86	15,00	1 ml in 1 L
mod. E	KH ₂ PO ₄	32,00	136,09	235,15	6,40	5 ml in 1 L
		$\mu\text{g/l}$		nMol	mg/l	
mod. F	FeCl ₃ ·6H ₂ O	100,00	270,30	369,96	50,00	0,18
mod. G	Na ₂ EDTA·2H ₂ O	200,00	372,24	537,29	100,00	2 ml in 1 L
	H ₃ BO ₃	185,00	61,83	2991,98	185,00	2,99
	MnCl ₂ ·4H ₂ O	415,00	197,90	2096,97	415,00	2,10
	ZnCl ₂	3,00	136,29	22,01	3,00	0,02
	CoCl ₂ ·6H ₂ O	1,50	237,93	6,30	1,50	1 ml in 1 L
	CuCl ₂ ·2H ₂ O	0,01	170,48	0,06	0,01	0,00
	Na ₂ MoO ₄ ·2H ₂ O	7,00	241,05	28,93	7,00	0,03
		mg/l		mMol	g/500 ml	
mod. H	NaHCO ₃	150,00	84,01	1,79	15,00	5 ml in 1 L

A, B, C, D and G are added to medium before sterilisation. E, F and H are added after sterilisation. H is sterilised in a closed bottle to prevent escape of CO₂.

checked by stt

Figure 12. Composition of the nutrient solution used as benchmark during the toxicity tests.

The outcome of the experiment is based on the change of transpiration in the trees between the treatments. Willows for the first experiment were delivered from the willow plantation at DTU Risø and cut manually outside DTU building 101 for the second one. After collection, they were measured with a caliper (Vernier, USA) for diameters 10-14mm, cut into lengths of 40cm and placed upright in a bucket with water 10cm up the stem in a south-facing windowsill until root and foliage growth was observed (approximately one week for roots and two weeks for the foliage).

After two weeks, the willows were weighed with an electronic balance of 0.001g accuracy (Mettler Toledo, Columbus, USA) and put in 500ml Erlenmeyer flasks filled with 400 ml of modified ISO8692 solution, diluted 1:1 with de-ionized water to halve the solution concentrations. The flasks were then covered with aluminum foil to minimize light seepage and reduce algae growth, while openings were clogged with aluminum foil and sealed with laboratory film (parafilm PM-996, amcor, USA) to ensure water loss was solely from transpiration and no evaporation would take place. The flasks were weighed with the balance and placed uniformly on a lighting table with 10 overhanging Philips TL-D 36W/840 light tubes (Philips, NL) delivering 3100 lm, each spaced 20 cm apart. As the middle light tube was defect, the flasks were placed on either side to ensure uniform lighting conditions. The placements of the flasks were marked on the table with a whiteboard marker as well as the stems of the trees in the facing directions.

Plants were weighed with flasks every morning, and daily weight/transpiration loss was noted. After 7 days, the average transpiration was calculated, and plants were ranked from most to least transpiring. The bottom 5 were discarded. The remaining 25 were weighed without bottles to determine biomass growth, and were then put back in the bottles, refilled with solution based on their transpiration. 5 treatments were employed in total: the top 5 were put in Nutrient Solution (NS) as control, the 5 second most transpiring in Tap Water (TW), the middle 5 in pure digestate (D100), second least transpiring in diluted digestate (DD) and the 5 least transpiring in nutrient solution mixed with digestate (DNS). Care was taken to put plants facing the exact same directions, and be in the same place by use of the markers on the tables and the stems, to decrease variability. The plants were then weighed daily Monday to Friday for two weeks, and the weight loss was noted each day. After two weeks, a clear trend in transpiration between the treatments could be seen, and the experiment was terminated. The trees were weighed without bottles, and leaves and roots were harvested. Solutions were sampled into 50ml propylene falcon tubes for analysis.

Fertigation strategy with permeate from co-digestion of FW and MWW

The first permeate dilutions were based on matching the nitrogen concentration to that of the nutrient solution. Having a total nitrogen concentration of 31.8 mg-N/L compared to 19.76mg/L in the nutrient solution, the permeate was diluted by 2 parts DIW to one part permeate for the diluted digestate treatment (DD), rendering a nitrogen concentration of approximately equal amount (21.2 mg-N/L). The nutrient solution with digestate mix was selected as 50/50 diluted nutrient solution and diluted digestate 2:1, rendering a concentration of 20.48 mg-N/L. The pure digestate treatment (D100) was chosen to determine possible toxicity effects of the permeate, and tap water (TW, treatment 4) as a nutrient-less baseline.

Fertigation strategy with permeate from co-digestion of BWW and MWW

The second digestate of MWW/BWW was diluted based on its salinity content to limit toxic effects. For the diluted digestate treatment (DD), the digestate was therefore diluted with two parts of water, reaching an EC of 7.12 mS/cm. Given the limit value of 8 for salt tolerant willow species, and a value of 5 as the limit for most species, the dilution was chosen to maximize nutrient content as well as to test salinity resistance. The nutrient solution-digestate mix (NSD) was chosen as 3 parts diluted NS (1:1) to one part of digestate rendering an EC value of 5.32 mS/cm, to maximize nutrients but limit salinity effects. The pure digestate treatment was again chosen to determine possible toxicity effects of the permeate and tap water as a nutrient-less baseline.

Pepper experiment

13 pepper plants of the breed Capsicum Annum, cv. Padrón peppers, 8 weeks in Ø10cm pots, were bought 01/05 from Høsterkøb Gentofte. Additionally, two control pots and a bag of standard



potting mix (described in section 0.2.2.1) were bought to keep evaporation controls and 7 for later repotting. At DTU, the plants were placed in plastic trays in a room with 10 light tubes (Philips TL-D 36W/840, Philips, NL) of 31000lm light, spaced 20cm apart, on a schedule of 16 daylight hours and 8 night hours. The temperature and humidity were uncontrolled, but at an approximately constant level of 24°C and 30% humidity. The control pots were filled with the potting solution and placed in-between plants to ensure similar levels of canopy shade and topsoil temperature. All pots were then weighed with an electronic balance of 0.001 accuracy (Mettler Toledo, USA) and one plant was set aside for determining the watering schedule.

Field capacity, wilting point and watering schedule for the peppers were based on theory of pepper irrigation from. The maximum available water level in the soil, determined as the soils field capacity (FC), is calculated in each pot, which is then given a water level for which it cannot drop below, determined as a 40% loss in total available water.

$$WL[g] = FC - 0.4 \cdot AW = FC - 0.4 \cdot (FC - WP)(3)$$

Here, WL [g] is water level at which the plant should be watered, and field capacity (FC) [g] is weight of the soil when it is holding the maximum amount of water, after excess water has percolated out to gravity. Available water (AW) [g] is the difference between field capacity and wilting point (WP), the point at which no water is available for plant uptake and the plant will die. To determine the field capacities, each plant/pot was thoroughly watered until water started seeping from the bottom, done three times to ensure the soil was fully saturated, i.e. both the micropores and macropores in the soil were completely filled with water.

The pots were then left to drain for 24 hours on a thin layer of leca balls in the bottom of the plastic crates, after which excess water had percolated, and the soil was at field capacity. For finding the permanent wilting point, one plant with soil was dried in a drying oven (VWR/memmert, Germany) at 105°C for 24h, after which it was put back in the pot and its weight was noted. Available water was calculated as the difference between the field capacities and the wilting point, and an allowable water depletion of 40% was determined. Plants were weighed each day and once coming close or below water levels (approximately every 3d day), all pots as well as controls were watered through light dripping on the topsoil with a 100ml syringe (Chiruna, Slovakia), bringing them as close to field capacity as possible.

Three treatments were implemented, namely full-strength Nutrient Solution as control (NS), digestate-nutrient solution mix (NSD) and diluted digestate (DD). Based on permeate 2, the permeate was diluted by 1:9 in the diluted digestate treatment (DD) to avoid salinity stress in cultivars, giving it an EC of 2.2mS/cm. The same dilution was chosen for the NSD treatment, at 1 part pure digestate and 9 parts NS, upped to 1 part digestate to 4 parts NS after 2 weeks.

As the peppers were grown inside, they had to be pollinated manually, done by poking the flower lightly with the finger to ensure pollen was released from the anthers of the flower and transferred on to the stigma. The process was done simultaneously with weighing each day during flowering. At the end of experiment (6 weeks), peppers were uprooted. Shoots, roots and fruits were separated and fresh mass recorded on a 0.001g precision balance (Mettler Toledo, USA).

Model for pollutant uptake of peppers

Pepper uptake of ionizable pharmaceuticals and personal-care products (PPCPs) found in the effluent was simulated with an analytic spreadsheet model. The model consists of two versions, with the classical/standard method, developed for neutral organics, considering partition processes and



unidirectional water flux from roots to leaves/fruits within the xylem (the central tissue responsible for transport of water/soil nutrients), setting the diffusion end point between root cells and water. Most PPCPs are however ionized at environmental pH, and an extended model was thus developed, taking into account (i) acid–base dissociation, (ii) diffusion across cell membranes for ions, (iii) protein sorption in plant compartments (roots, leaves, fruits), and (iv) bidirectional phloem flux, i.e. from leaves to fruits and leaves to roots. Only the PPCP version of the model considering the ionizable PPCP's is reported here (Figure 13).

All equations were solved analytically and are implemented in a publicly available Excel workbook where users can supply compound-specific constants $\log K_{OW}$, pK_a , H , K_{AW} , K_{HSA} , and site-specific parameters such as soil concentrations, pH, and crop specifics.

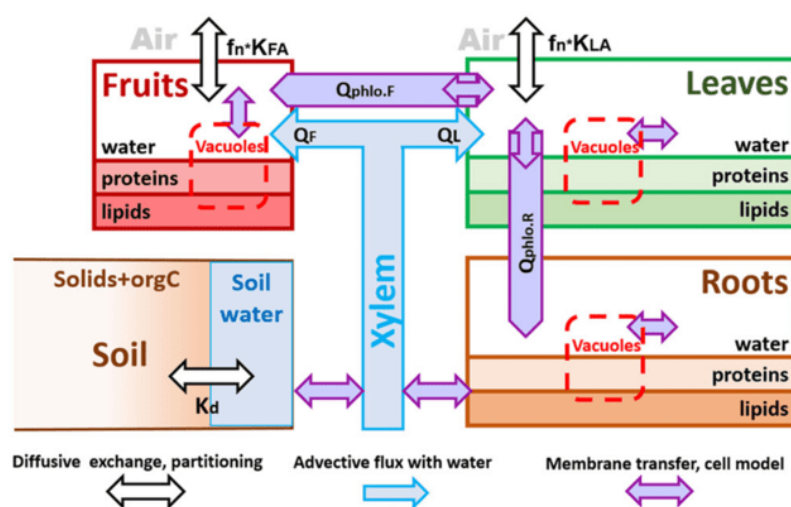


Figure 13. Processes for consideration of ionization and transportation of CEC in PPCP model

• Results for the reuse of treated water through fertigation

Three fertigation tests were conducted: 1) hydroponic cultivation of willow trees (energy crop); 2) pot cultivation of Padrón peppers (edible crop); 3) greenhouse cultivation of spinach (edible crop). The first two compare standardized fertilizers with the permeate from the AnMBR, while the third compares the permeate with effluents from tertiary treatment of municipal wastewater.

Willow transpiration

Two fertigation trials were conducted with willow trees, using effluent from two different periods 5 (MWW/FW) and 7 (MWW/BWW). Both trials applied different mixtures and dilutions of digestate compared against a standard chemical nutrient fertilizer, show that reclaimed water induces an initial stress to the willow trees, but eventually yields better growth compared to tap water, giving best results when mixed with conventional fertilizers due to the different nitrogen forms in reclaimed water and standard chemical nutrient fertilizers. Figure 14 shows the hydroponic set up used for both willow tree experiments.



Figure 14. Hydroponic willow tree experiment set up

Figure 15-Figure 17 show the relative and absolute transpiration rates for both fertigation experiments.

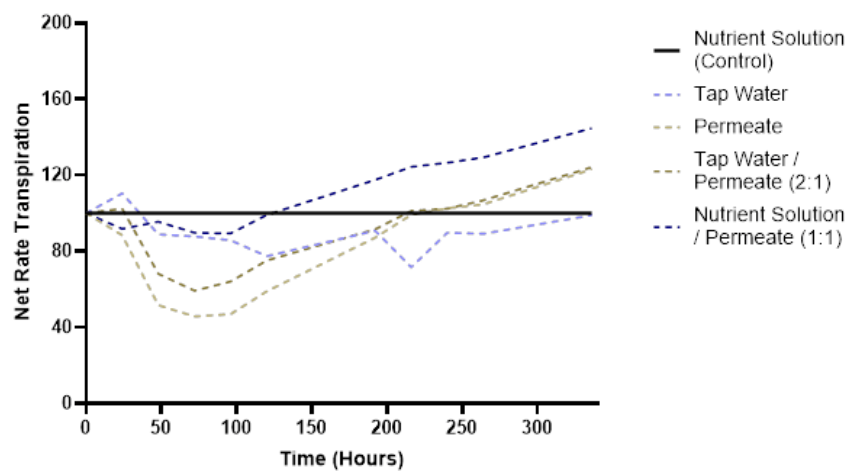


Figure 15. Transpiration rates in primary willow fertigation experiment. willows fertigated with nutrient solution, tap water, digestate, a diluted digestate, and a 1:1 nutrient solution and digestate mix

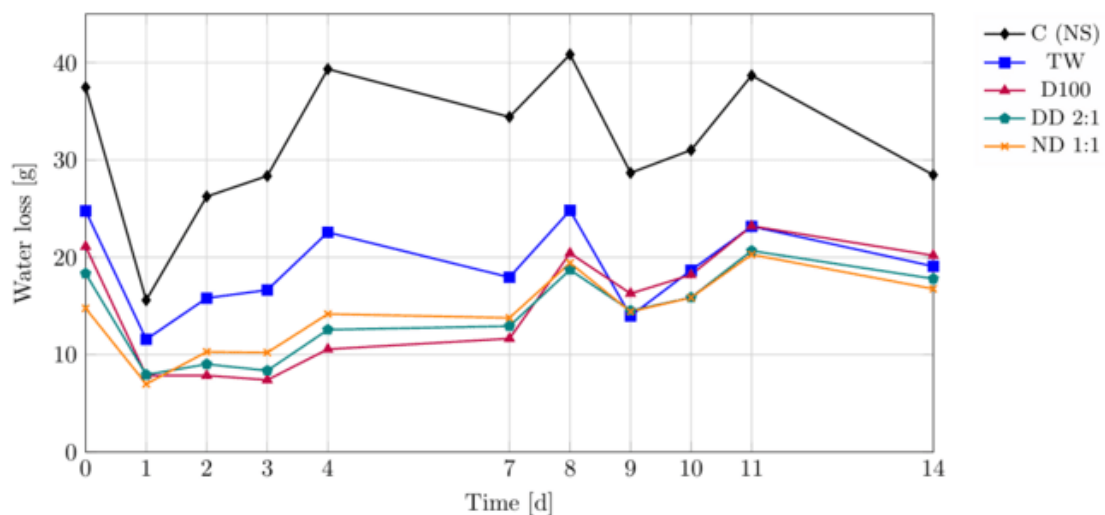


Figure 16. Absolute transpiration rates in primary willows fertigation experiment with nutrient solution (NS), tap water (TW), digestate (D100), a diluted digestate (D.dil. 2.1), and a 1:1 nutrient solution and digestate mix (Nut. + Dig. 1.1)

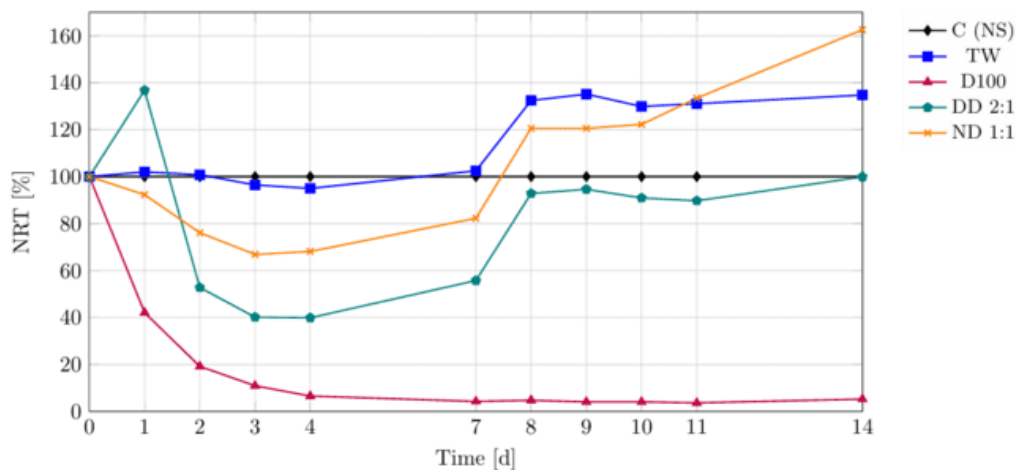


Figure 17. Transpiration rates in secondary willows fertigation experiment with nutrient solution (NS), tap water (TW), digestate (D100), a diluted digestate (D.dil. 2.1), and a 1:1 nutrient solution and digestate mix (Nut. + Dig. 1.1)

The depth of the early NRT depressions scaled with the initial ammonium loads and osmotic jumps, whereas the long-term performance seemed mainly dependent on how much assimilable N ($\text{NH}_3+\text{NO}_3^-$) solutions were delivered. The NSD mixtures, offering $\text{NH}_4^+/\text{NO}_3^-$ ratios below 1 and moderate salinity, delivered the most sustained transpiration, and by implication, growth potential. Nitrate-ammonium fertilization has on several occasions been confirmed to prove the most balanced nitrogen uptake, as the combination of both ions act synergistically for nitrogen uptake. Plants synthesize NO_3^- by step wise reduction into ammonium, requiring significant amounts of energy supplied by photosynthetic products. Conversely, ammonium, can be directly synthesized into amino acids, and conserves metabolic energy by bypassing the nitrate reduction steps. Growth promotion triggered by an appropriate $\text{NH}_4^+/\text{NO}_3^-$ ratios has been coupled to accumulation of more carbon and nitrogen, a higher leaf area and photosynthetic rate, higher chlorophyll concentration, increased auxin synthesis, improved absorption of nutrients, improved root growth and a lower root/shoot ratio. Trials exploring $\text{NH}_4^+/\text{NO}_3^-$ ratios report 10% additional biomass growth in willows, and over 100% additional gains on centipede grass as well as increased nitrogen-use efficiencies at $\text{NH}_4^+/\text{NO}_3^-$ ratios near 1 compared to either ion alone. The use of co-digested MWW with FW as a fertilizer was not found explicitly in literature, but the use of ammonium rich digestates, or anaerobic digestate of food waste by itself, has been examined on numerous occasions.

Pepper experiment

The pepper plant cultivation tests were conducted in pots as shown in Figure 18.



Figure 18. In-pot pepper plant experimental set up.

Figure 19 and Figure 20 show both the relative and absolute transpiration observed throughout the experiment.

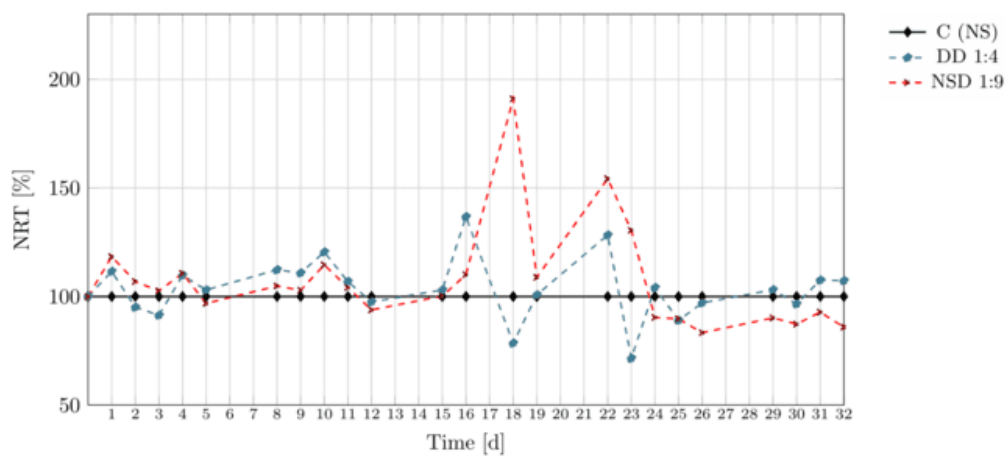


Figure 19. Normalized relative transpiration (NRT) peppers from period 7 digestate

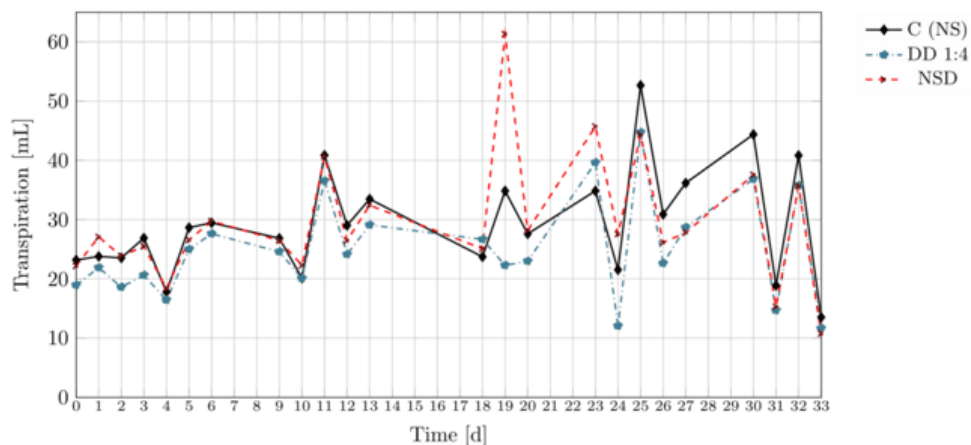


Figure 20. Absolute transpiration of peppers.

Figure 21 shows the water use efficiency (WUE) of the various components of the pepper plants and each fertigation mixture.



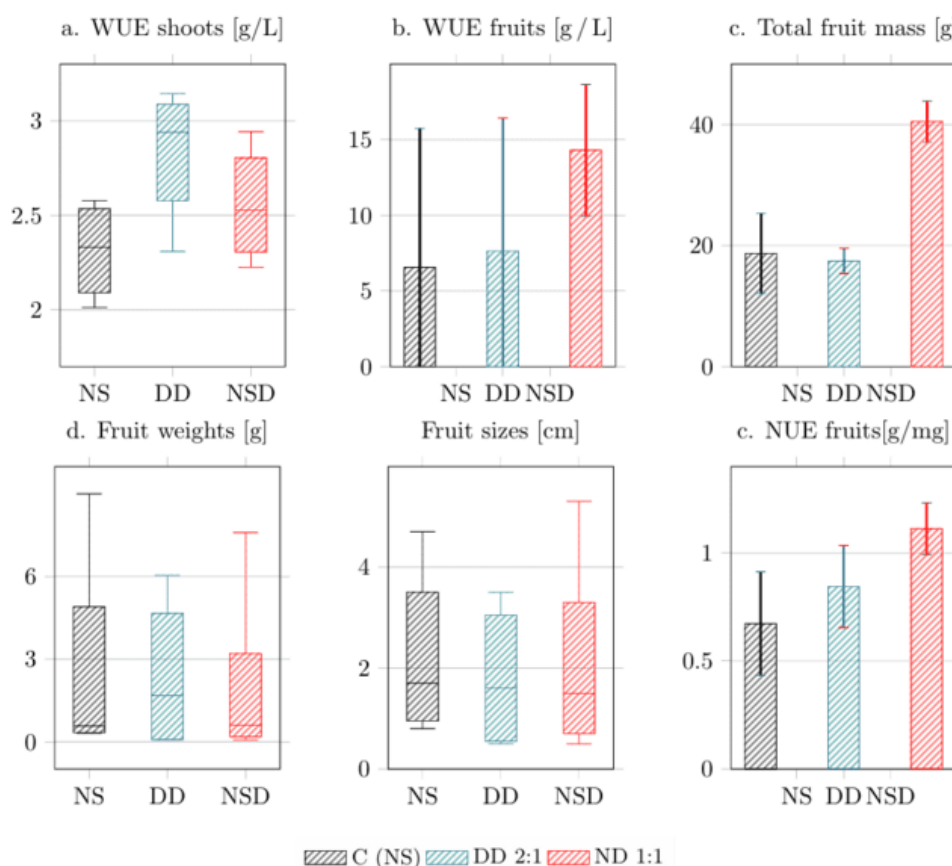


Figure 21. Padron peppers fruit mass as a function of water used in nutrient solution, diluted digestate and a combination of both

In sum, the digestate dilutions did not seem to toxify but rather nurture, as confirmed by the NSD and DD solutions both yielding higher shoot/fruit WUE and NUE, while the NSD treated peppers accumulated the highest fruit mass and saw best performance overall.

The DD not inducing a toxicity response over the 6-week period as well as maintaining similar levels of transpiration and fruit yield as the DNS/NS treated peppers, indicate the BWV/MWW permeate was beneficial when applied as additional fertilizers for the peppers in the experiment, and can be applicable as fertilizer when diluted.

CEC Uptake prediction model

Table 50 shows the estimation of CEC plant uptake predicted by a model for edible crops when irrigated with reclaimed water. The model simulates root uptake of CECs from the permeate, considering cell-membrane diffusion, translocation to fruits and leaves, and other plant physiology considerations.

Table 50. Model prediction of CEC uptake and concentration in Padron peppers

		Acetaminophen	1H-benzotriazole	Metoprolol	5-methyl-1H-benzotriazol	Venlafaxine	Losartan	Hydrochlorothiazide
ng/kg fw	Roots	744.5	45.9	45.3	4.3	2.4	50.4	234.1

		Acetaminophen	1H-benzotriazole	Metoprolol	5-methyl-1H-benzotriazol	Venlafaxine	Losartan	Hydrochlorothiazide
ng/kg fw	Leaves	1213.9	88.5	45.3	4.1	2.1	10.5	484.7
ng/kg fw	Pepper	2082.1	179.0	29.9	2.9	1.4	10.8	1003.5

When evaluating the model outputs, the conclusion was that pharmaceutical uptake into pepper fruits was extremely low for all compounds studied. The model suggested that cationic substances such as metoprolol and venlafaxine to a larger degree to be confined to roots and less so in fruits. Polar compounds like acetaminophen were somewhat more mobile, but as it is shown to degrade rapidly both in the soil and in the plant, its predictions could be deemed exaggerated. Benzothiazoles also exhibiting degradation in water and sunlight further emphasizes the need for development of degradation rates for more accurate model predictions.

Nevertheless, diluting the wastewater (1:5) ensured pollutant loads to be very low, diminishing any serious accumulation in edible tissues for any compound. Uptake in plants is roughly proportional to exposure concentration, so such low inputs yield extremely low residues in plant tissues. The model-predicted pepper fruit concentrations would quite possibly be below analytical detection limits or only in the picogram to low nanogram per gram range. Fruit contaminant levels are as such likely beneath health concern thresholds by several orders of magnitude, given that even much higher exposures produce negligible risk quotients in crop intake studies. Improved data on degradation rates, protein-sorption coefficients and measured soil concentrations rather than estimates would have provided a more realistic uptake scenario.

No current specific regulatory limits exist in the EU or DK, however all predicted concentrations are significantly lower than typical food safety thresholds for unregulated residues. However, considering the low effluent concentrations as well as its dilution, the predicted pepper fruit residues are likely far below any known ecotoxicological effect thresholds and present no tangible risk to consumers or the environment.

Irrigation of spinach

Alongside DTUs lab experiments with willow trees and Padron peppers, on-site trials at Svaneke WWTP were carried out to investigate the effects of fertigation on spinach plants (Figures 22, 23, and 24). The greenhouse and irrigation system were established by BEOF and GARD in spring 2025. The experimental setup consisted of a tunnel greenhouse, irrigation tanks, and drip hoses. Inside the greenhouse, two rows of spinach plants, each containing six boxes with six plants per box, giving a total of 36 plants per row.

Row 1: Irrigated with SYMSITES reclaimed water (permeate from the AnMBR).

Row 2: Irrigated with conventionally treated wastewater that had undergone slow sand filtration prior to use. This slow sand filter was developed and piloted under the WATERMAN project, funded by the INTERREG Baltic Sea Programme. More details can be found here: [Euroregion Baltic | Recycling treated wastewater for agricultural use](#)

The WATERMAN-treated water contained relatively low nutrient levels compared to the nutrient-rich SYMSITES water. The aim of the trial was to compare plant growth responses between the two irrigation sources. However, significant clogging issues—particularly in the SYMSITES irrigation



line—prevented the system from functioning properly. The clogging could have been caused by mineral precipitation, possibly calcium carbonate (Danish wastewater is relatively hard), which accumulated inside the irrigation hoses. The exact cause of the clogging was not investigated further as it was out of scope of this project. As a result of the clogging, the experiment could not be completed as originally planned and had to be terminated in July 2025.

Table 51. Comparison of Waterman vs Symsites permeate used for fertigation of spinach plants

Parameter	WATERMAN mg/l	SYMSITES mg/l
	Average values April – June 2025	Average values - April – June 2025
Ammonium	1,68	30,19
COD	22,36	145,5
Nitrate	0,25	13,42
Tot P	1,02	3.76
Tot N	2,75	63,92

Despite the early termination, initial observations revealed clear differences in plant development between the two treatments. Spinach irrigated with SYMSITES water produced larger leaves, broader plants with dark green colours, whereas those irrigated with WATERMAN water developed smaller leaves and exhibited a taller, thinner growth pattern and the leaves were light green. These differences are illustrated in Figure 24. This is expected, since Symsites permeate had higher nutrient content (Table 51).





Figure 22. Polytunnel and feed tanks for irrigation experiments



Figure 23. Spinach plants irrigated with SYMSITES permeate and WATERMAN effluent (April)



Figure 24. Spinach plants irrigated with SYMSITES permeate (Row 1 -LEFT) and WATERMAN effluent (Row 2-RIGHT), Photo from June 2nd, 2025

5.2.2 Char for beer mash

In this ecosite it has been difficult to select a suitable waste to be able to be pyrolyzed. The brewery has two types of “semi” solid wastes:

- Yeast waste
- Beer mash waste (spent grain)

However, finally it was decided to only characterize spent grain, since yeast waste was not solid, and very foamy of nature. Figure 25 shows that there is a lot of liquid in the bag as if it had come out of the container (left). The spent grain comes quite wet (middle), and we have taken a sample of the liquid to also analyse it (only applicable techniques) (right).





Figure 25. Sample waste bag (left) beer mash (center) and liquid (right) received.

Table 52 contents the results of humidity, volatile at 550 °C, density and LHV of the beer mash waste as preliminary analysis.

Table 52. Physic-chemical characterization of meat industry waste.

Sample	Humidity (%)	Volatile at 550 °C (%)	Density(kg/m ³)	LHV (cal/g)
Beer mash (solid)	84.4	96.9	139.4	4107.19
Beer mash (liquid)	95**	/*	/*	/*

*Not apply. ** 2.14 g/L solid in suspension

As it is observed this type of waste has high humidity content (84%), which is not beneficial since the dry step cannot be avoided. In this case extra dry step will be needed. Volatile compounds are high ~ 97%. Density and LHV do not fit the requirements of pyrolysis reactor (industrial point of view), but we will see the rest of characterization of the sample.

Table 53 summarizes ion chromatography and analysis elemental results.

Table 53. Ion chromatography and elemental analysis results.

Sample	Halides			Elemental analysis				
	Fluorides (ppm)	Chlorides (ppm)	Bromides (ppm)	C (%)	H (%)	N (%)	S (%)	O (%)
Beer mash (solid spent grain)	65.18	214.92	0	47.7	4.5	3.5	0.11	42.6
Beer mash (liquid)	335.36	244.78	<8	/*	/*	/*	/*	/*

*Not apply

Results in Table 53 shows that fluoride and chlorides content in these samples are between 60-300 ppm and bromides content is quite low or zero. Elemental analysis results show a carbon content around 48 %, and with the rest of elements (H, N, O, S) the sum is near 100, thus ash percentage will be low (see TG analysis).

These wastes have also been analysed by ICP and the results are summarizing in Table 54.



Table 54. ICP analysis of sample beer mash waste.

Metal	Concentration (ppm)	
	Solid	liquid
Ag 328,068 (A)*	4,3	< 0,0662
Al 396,152 (A)	300	2,08
B 249,773 (A)	19,4	< 0,271
Ba 233,527 (A)	12,3	0,806
Bi 223,061 (A)	< 16,6	< 0,618
Ca 317,933 (A)	1912	> 302
Cd 214,438 (A)	< 0,711	< 0,0264
Co 228,616 (A)	4,35	0,0774
Cr 267,716 (A)	6,3	0,0827
Cu 324,754 (A)	25,1	0,538
Fe 259,941 (A)	321	6,08
Ga 417,206 (A)	< 5,68	< 0,211
In 230,606 (A)	< 21,5	< 0,800
K 766,491 (A)	1796	227
Li 670,780 (A)	< 1,59	< 0,0592
Mg 280,270 (A)	1396	258
Mn 257,611 (A)	23,4	1,47
Na 589,592 (A)	291	71,4
Ni 231,604 (A)	3,24	0,106
Pb 220,353 (A)	< 11,2	< 0,417
Si 251,612 (A)	442	43,5
Sr 407,771 (A)	27,5	4,51
Tl 190,864 (A)	5,66	0,067
Zn 213,856 (A)	69,4	5,84

*Number (A)= means the wavelength used to analysis the corresponding metal.

From Table 54, the main metals present in solid sample are Ca and Mg (beer mash micronutrients). The rest of elements are not present in representative quantity. In liquid sample, the elements are in lower concentration as was expected, since they are diluted in the water.

Finally, TG analysis was obtained. Figure 31 shows the TG curve obtained.



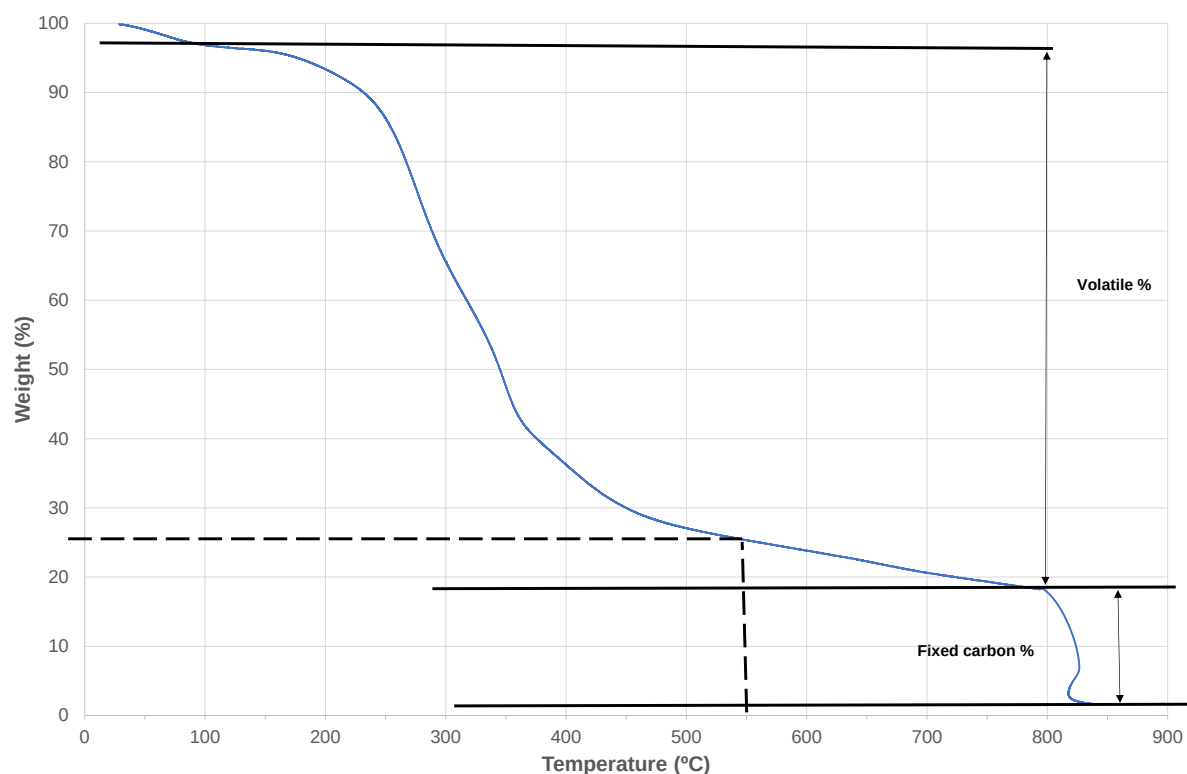


Figure 26. TG curve analysis of beer mash waste

From TG analysis the following results have been obtained (Table 55).

Table 55. ICP analysis of sample beer mash waste.

Immediate analysis						
Sample	Humidity (%)	Volatile (%)	Fixed (%)	Carbon	Ash (%)	% at 550 °C
Beer mash	3.2	78.5	16.5		1.7	25

From TG analysis it is confirmed that the ash percentage in this sample is low (~2 %) and the remain solid fraction at 550 °C is about 25 %. The humidity after dry step (24 h at 110 °C) it is good. This material could be an adequate candidate to obtain char however the raw material had 85 % of humidity an extra step of dry the sample could be added which increase the costs (CAPEX and OPEX) and the energy used of the possible pilot plant.

Thus, this material could be an adequate candidate to obtain char however the raw material had 85 % of humidity an extra step of dry the sample could be added which increase the costs (CAPEX and OPEX) and the energy used of the possible pilot plant.

5.2.3 Upscaling the AnMBR system-mapping the waste streams

Below is an overview of the yearly quantities of waste streams on Bornholm that potentially could be used in an upscale scenario.



Table 56. Yearly quantity of waste streams on Bornholm (2025)

Feed streams	Yearly Quantity	COD mg/l
Municipal wastewater	5-7.000.000 m ³	250
Food waste	3000 tonnes	90000
Brewery wastewater	5.000-10.000 m ³	7500
Dairy wastewater (whey)	73000m ³	60000
Slaughterhouse wastewater	135.000 m ³	4000
Pig and Cow Manure	600.000 tonnes	40000

- *Municipal Wastewater*

Municipal wastewater has significant potential for irrigation, but its biogas yield per cubic meter is low due to the low COD content. Utilizing municipal wastewater in an Anaerobic Membrane Bioreactor (AnMBR) adds water (irrigation potential) to the system but also requires substantial energy for heating, resulting in high treatment costs.

- *Food Waste*

Food waste has a high COD content, leading to a high biogas yield. Currently, food waste on Bornholm is collected and transported to Zealand under a 10-year contract with Gemidan.

- *Brewery Wastewater*

Brewery wastewater is currently treated at Bornholms Bioenergy A/S (Bigadan), which operates a pig and cow manure biogas on Bornholm.

- *Dairy Wastewater (Whey)*

Dairy wastewater, specifically whey, is currently used as animal feed. It has a high biogas potential.

- *Slaughterhouse Wastewater*

Slaughterhouse wastewater is sent to the Rønne Wastewater Treatment Plant (WWTP). Some fractions from the slaughterhouse are sent to Bornholm Bioenergy A/S.

- *Pig and cow manure*

Bornholm Bioenergy A/S treats around 110.000 tons of manure per year. There are plans to expand this facility to treat up to 400.000 tons per year.

- *Regulatory implications of mixing feed streams (manure)*

In Denmark, the Sludge Order regulates the use of sewage sludge on farmland, including limits for contaminants and requirements for sanitation before application. If sludge is mixed with manure, the entire mixture must still meet all sludge related rules on treatment, documentation, and use.

The Arla directive is significantly stricter than Danish law. Arla (Danish multinational Dairy company) completely prohibits the use of sewage sludge on any fields connected to Arla milk production. This ban also covers mixtures, meaning that manure blended with even small amounts of sludge cannot be used on Arla supplying farms.



Sanitation remains a core requirement under Danish regulation, typically achieved through digestion, composting, or thermal treatment to reduce pathogens. However, for Arla suppliers, sanitized sludge is still not permitted in any form.

5.2.4 Potential applications of the generated stream on Bornholm

Treated water

Table 57. Potential application of the generated streams (treated water)

Identification of potential applications of treated water at the Danish Ecosite, Bornholm	Estimated yearly need	Quality required / applicable regulation
Agricultural Uses Farming (pigs, cows etc.): 5000 cows, 500.000 pigs per year Irrigation of crops, 30.000 Ha.	350.000 m3 per year 0 – 21.000.000 m3 year, seasonal April-July	Drinking water quality standard + permission at The Danish Veterinary and Food Administration EU regulation 741/2020, Class A, B, C, D.
Municipal Uses Irrigation of parks, recreational areas and football fields	N/A, April-September	EU regulation 741/2020, Class A
Industrial Uses Feed for ultra-pure water for PtX (Power-to-X) production Cooling water	1.750 m3 per 1 MW electrolyser per year Baltic Energy Island project: 800 MW = 1.400.000 m3/year N/A	Ultra-pure water: Typically < 1 µS/cm, depends on electrode type. Needs additional treatment, e.g., reverse osmosis or membrane distillation. N/A
Other Ammonia stripping from the treated water	N/A	N/A



Sludge

Table 58. Potential application of the generated streams (sludge).

Identification of potential applications of sludge at the Danish Ecosite, Bornholm	Estimated yearly need	Quality required / applicable regulation
Agricultural Uses: Conventional farms	Almost 30.000 hectares are cultivated on Bornholm, but application is limited to land not used when connected to milk products.	The Danish Sludge order defining quality requirements for sludge on agricultural land.
Struvite production/ammonia stripping	N/A	Danish Fertilizer and Soil Improver Regulation (Gødnings- og jordforbedringsbekendtgørelsen)
Biochar production through pyrolysis	N/A	Danish Fertilizer and Soil Improver Regulation (Gødnings- og jordforbedringsbekendtgørelsen)

Biogas

Table 59. Potential application of the generated streams (biogas).

Identification of potential applications of Biogas	Estimated yearly need	Quality required / applicable regulation
Industrial uses: Process energy (Jensen Denmark, Hasle Refractories, Beck, Victor Vask, Svaneke Bryghus) Heavy transport (trucks etc.)	Svaneke Bryghus: Yearly need of 50225 kg (700.638 KWH)	The Danish gas quality regulation BEK no. 230 21/03/2018 <u>Bekendtgørelse om gaskvalitet</u>
Upgrading methane to biofuel etc.	N/A	N/A



5.2.5 Upscaling the industrial Symbiosis at Svaneke WWTP and use of the HVNR

A full-scale industrial symbiosis scenario has been developed and evaluated, integrating municipal wastewater, brewery wastewater, and food waste streams. The primary objective is to maximize biogas production, generate reclaimed water for agricultural irrigation, and establish a circular resource flow among local stakeholders.

Feedstock Definition and Annual Quantities

The scenario includes the following input streams:

- **Municipal wastewater:** 33,000 m³/year (approximately 6% of total inflow to Svaneke WWTP)
- **Brewery wastewater:** 6,000 m³/year (representing 100% of the locally available resource)
- **Food waste:** 3,000 tonnes/year (representing 100% of the locally available resource)

The selected volumes are based on the irrigation demand at Frennegårds (GARD), ensuring that the reclaimed water production matches realistic storage, distribution, and utilisation capacities. The scenario further reflects a maximum resource recovery approach by incorporating the entire available volumes of brewery wastewater and food waste.

The projected annual biogas production is approximately 156,000 m³ of methane. Treated water production is estimated at 40,000 m³ per year.

The energy balance shows an output of 1320 MWh and an input of 1145 MWh, resulting in an energy self-sufficiency of +15% if all gas is used for heating and electricity internally. Energy consumption for biogas upgrading storage and transport, is not included. Using all energy for internal use is however not in line with the symbiosis – outputs should be utilized in other industries.

Potential Uses of Biogas

Svaneke Bryghus (BRYG) consumes 50,000 kg of propane gas annually, which can be fully supplied by the biogas produced from the symbiosis. An additional 106,000 kg of gas could be supplied to other local industries. However, in such a scenario the AnMBR energy demand must be covered by other sources. It's important to note that the biogas from the AnMBR must be upgraded by removing CO₂, H₂S, and water.

For detailed analysis of the **biogas** water please refer to D4.3.

Potential Uses of Treated Water

Frennegård (GARD), a local farm, has 100 hectares equipped with irrigation systems and an annual water demand of around 40,000 m³ - the actual demand can fluctuate depending on rain fall, but in the last 10 years drought occurs more often during the months of April to August. The symbiosis could fully meet GARD's irrigation requirement in most years. However, currently GARD only has storage capacity for 7,000-10,000m³, but they have plans to expand the reservoir in the future to 30,000-40,000m³. Currently, the reservoir is used for collecting surface and rainwater for irrigation.

For detailed analysis of the **treated water** and **sludge** please refer to D4.3.

Sustainability modelling scenarios-data from PRJ

The benchmark scenarios are developed based on the system expansion approach. In LCA methodology, dealing with multifunctionality where a process or system produces multiple outputs is



done through system expansion approach. Under this method, system boundaries are broadened to incorporate the alternative processes that would otherwise be required to deliver the secondary outputs generated by the process.

The benchmark scenario serves as a reference system for assessing the environmental performance of the EcoSite. It is based on the operational conditions of period 3. The analysis is done considering the operating conditions, under which the facility treats 250L/day each, municipal and brewery wastewater, while organic waste is not yet incorporated into the process. The Danish EcoSite generates 500L/day of treated water and 0.55m³/day of biogas. Accordingly, the benchmark scenario evaluates conventional wastewater treatment, which primarily produces treated water. Biogas production process is also included in the benchmark system to allow for a comprehensive comparison of the environmental impacts associated with all outputs. The treatment process of the Danish EcoSite is shown on the left side in Fig. 14, where wastewater serves as the primary input. The system produces treated water and biogas. To allow for a fair comparison, the benchmark system is expanded to generate the same outputs as the Danish EcoSite. Conventional wastewater treatment processes are integrated. For biogas production, an alternative anaerobic digestion process using organic material is included. Since there is no sludge discharge (i.e. bio-fertilizer), it is excluded from the impact assessment, though it is retained in the schematic for completeness.

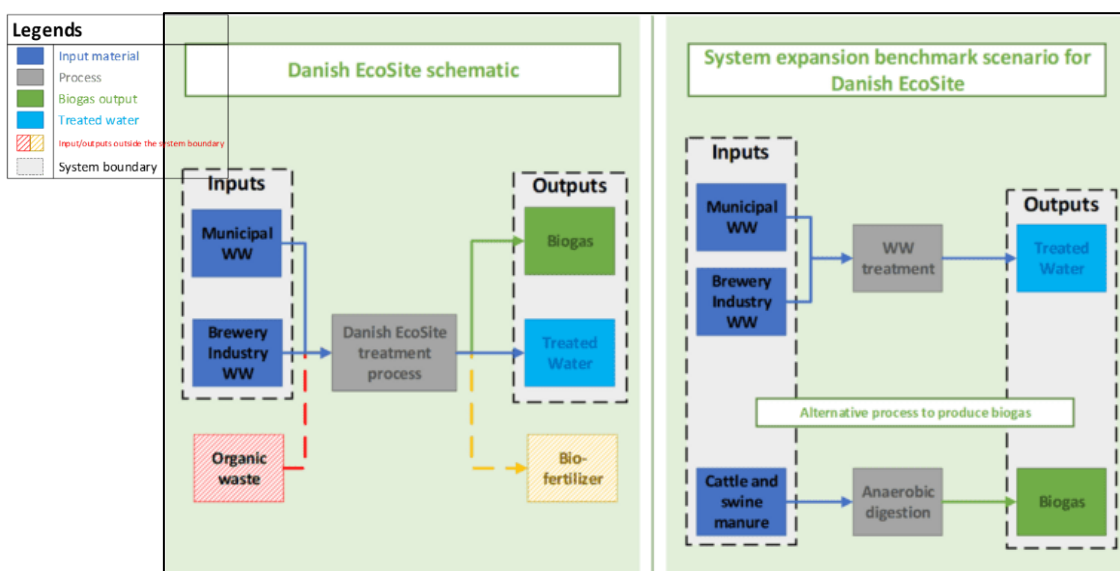


Figure 27. Ecosite schematic and system expansion diagram

Reduction of the GHG emissions to near-zero

Based on processes as described above the parameters considered for the analysis are mentioned below:

INPUTS:

250L/d of MWW and, **COD considered: 250mg/l**

250L/d of BWB, **COD considered: 7500mg/l**

OUTPUTS:

500L/d of treated water and,

0.55m³/d of biogas

The total energy consumption of the EcoSite is considered as approximately 86kWh per day which is calculated from the data log of energy consumption provided. For the COD mentioned above, the

datasets for treating WW with comparable CODs are selected from ECOINVENT LCA database. The alternative process to produce biogas is selected as anaerobic digestion process of cattle and swine manure. The alternative processes that match with quantity of inputs and outputs of the EcoSite to get the environmental footprints of conventional process considered as compared to the EcoSite. The data set for the energy consumption of the EcoSite was created based on the energy mix of the Bornholm which is 100% renewable (44% biomass, 34% wind and, 22% PV). Based on the inputs and their corresponding outputs, as mentioned above, the carbon footprints (GHG emissions) based on the LCA methodology accounted as:

EcoSite (kgCO ₂ eq.)	Benchmark (kgCO ₂ eq.)
1430 kgCO ₂ eq./year	1386 kgCO ₂ eq./year

Unfortunately, based on the methodology and consideration depicted above the GHG emissions of the Danish EcoSite are 43.70 kgCO₂eq./year higher than of the conventional methods which converted to 0.12 kgCO₂eq./day.

The Danish EcoSite shows lower energy efficiency compared to the other EcoSites. The combination of a relatively low functional unit and high energy consumption resulted on high GHG emissions when compared to the benchmark. Danish EcoSite consumes a significant amount of energy to process a limited volume of waste streams, generating minimal HVNRs (biogas). Being energy a primary driver of operational impacts of the EcoSite, it must be observed that climate conditions for Danish EcoSite play a relevant role in direct energy requirements per functional unit. Indeed, compared to other EcoSites, for Danish EcoSite extra energy is required to cover the outdoor environment temperature drops, especially in winter to guarantee temperature control of the AnMBR.

5.2.6 EU Regulation 2020/741

EU Regulation 2020/741 establishes minimum requirements for the safe use of reclaimed wastewater for agricultural irrigation. It came into force in 2023 and applies directly to all EU Member States unless they formally opt out. However, Denmark has decided to opt-out and does not allow wastewater reuse for irrigation.

Why Denmark Chose to Opt Out of EU Regulation 2020/741

Denmark decided in January 2026 not to adopt EU Regulation 2020/741 on the reuse of treated wastewater for agricultural irrigation. The Ministry of the Environment concluded that the regulation was not necessary under Danish conditions. Unlike many southern European countries, Denmark does not face structural water scarcity according to the Ministry, and the national need for reclaimed irrigation water is considered limited. Implementing the regulation would also require extensive monitoring systems, documentation procedures, and treatment upgrades, which the Ministry viewed as disproportionate to the expected benefits. Another factor behind the decision was concern about environmental and health risks. Danish authorities highlighted uncertainties related to micropollutants such as pharmaceuticals and PFAS, as well as the potential effect on groundwater sources. The Ministry stated that the decision will be revisited no earlier than 2029, once more research, pilot projects, and national risk assessments are available. Until then, treated wastewater—including AnMBR permeate—cannot be used for irrigation in Denmark. As a result of the Denmark's long decision time (since 2022) and their policy position on the EU regulation, we were unable to apply for water reuse at Frennegård. Instead, we established irrigation experiments on-site using spinach, a crop grown at Frennegård. In parallel, DTU conducted irrigation experiments with willow trees and Padrón peppers. Bornholm – climate and irrigation needs Bornholm has a mild



maritime climate shaped by the Baltic Sea, which limits temperature extremes and gives the island some of Denmark's highest sunshine totals. Summers are typically dry because the cool sea suppresses cloud formation and reduces convective rainfall. Despite being an island, rainfall increases slightly toward the island's higher central terrain, but summer precipitation remains modest. Drought Development (DMI Data, Last ~10 Years) DMI's drought index (0–10 scale) shows that Bornholm repeatedly reaches very high drought levels: **2018**: Bornholm hit index 10, the maximum value, marking the worst drought in 10 years at the time. **2024**: In June, Bornholm reached 9.8, placing it among the driest areas in Denmark. **2025**: In May, DMI reported Bornholm at 8.7, indicating a deficit of ~87 mm of soil water.

Across the decade, Bornholm consistently appears among the earliest and most drought prone regions in Denmark during dry summers, driven by high sunshine hours and limited summer rainfall. With rising temperatures and longer dry spells expected under future climate scenarios, Bornholm is likely to face more frequent and more intense summer droughts. Sandy soils in the island's south areas will experience the strongest moisture deficits. As a result, agriculture will require increasing supplemental irrigation to maintain stable yields, alongside improved water storage, efficient irrigation systems, and drought resilient cropping strategies.

- **Elaboration on key challenges encountered during pilot operation and their scaleup perspectives**

Pretreatment

According to the membrane manufacturer Alfa Laval, the membranes in the AnMBR require a pretreatment filter with a maximum pore size of 500 microns to prevent clogging between the flat sheet membranes.

Food waste, especially fibrous raw food waste, clogs the drum filter of 100 and 500 microns, leading to high reject rates. Coarse shredding of food waste results in significant loss of organic material in the reject.

Implementing a shredding solution that processes food waste into a very fine mass can help reduce the loss of organic material and improve filtration efficiency.

Introducing a self-cleaning pre-treatment filter that does not clog easily can enhance the overall efficiency of the filtration process. However, without a fine shredding solution, a self-cleaning filter would still create a lot of reject. Alternatively, food waste can be pretreated through pulping processes, which reduce the solid content by generating a bio-pulp highly biodegradable.

During piloting experiments a 2000-micron pre-filters were evaluated as well as the impact on the flat sheet membranes. The 2000 microns reduced the amount of reject significantly, but could potentially increase fouling in the membrane system, which was not observed during the experiments. This can be due to relatively short campaigns with food waste, without experiencing long-term effects on the membranes.

Other challenges to upscaling – Non-technical barriers

There are several challenges to upscaling the AnMBR system to cover the entire region of Bornholm. A few of these are listed below:

- *Current WWTP system and food waste to municipal wastewater ratio*



The availability of high-COD content waste, such as food waste, is limited. This is crucial because the energy efficiency of the AnMBR system relies heavily on the presence of high-COD waste to generate sufficient biogas (energy and heating).

- *Storage, Distribution and reclaimed water supply*

Determining how to store the treated water is a significant challenge. Treated water storage structures, such as service reservoirs and water towers, must be designed to prevent chemical contamination, microbial growth, evaporation and ensure water quality. The stored water can experience ammonia loss through evaporation, especially if exposed to air. This can lead to a decrease in ammonia concentration over time.

The treated water cannot be discharged into the Baltic Sea due to its high nitrogen content, which could lead to environmental issues such as eutrophication.

In other words, the stored water must be used for irrigation and therefore requires that there is an agriculture sector in need of water and that it is feasible for the sector to pay for the water.

- *WWTP Structure*

BEOF operates seven wastewater treatment plants with a decentralized structure. Upscaling would require seven AnMBRs, which is logistically complex and costly. Although there are plans to centralize the wastewater treatment system, a new centralized WWTP will not be ready until 2040 at the earliest. This delay poses a significant challenge to timely upscaling. Despite the challenges, decentralization can offer benefits. Small AnMBRs can treat local streams and supply water to nearby farms, providing a more localized and potentially more efficient solution. Furthermore, centralized systems will rely on long sewer systems and pipes for re-distribution of the treated water.

- Final considerations for use of AnMBR systems for reclaimed water
- Using big amounts of municipal wastewater as feedstock in an AnMBR system requires too much energy, primarily due to the high heating demand and low COD concentration.
- The treated effluent from the AnMBR contains nutrients (nitrogen and phosphorus) – the ideal use is irrigation. The treated water is not suitable for discharge into the Baltic Sea due to too high amounts of nutrients. In other words, supply and demand must be aligned.
- It could be more efficient to take irrigation water after the conventional biological treatment, since the water can be discharged to the Baltic Sea in case there is not enough demand. However, the nutrient content would be lower, demanding more conventional fertilizers.
- Use the AnMBR for high COD feed streams and only use wastewater for “dilution” to keep dry matter content within the membrane operational limits.



6 AUSTRIAN ECOSITE

6.1 Description of the Ecosite

The Austrian Ecosite focuses on the treatment of the WW from the meat processing industry and blood waste from the slaughterhouse as well as food waste from the school and kindergarten supplied by the companies catering (see diagram in Figure 28). The industry WW contains higher organic load than municipal wastewater and is used for biogas production. After a flotation process that reduces the volume of the primary stream, the flotation sludge is fed into the biogas reactor. As a co-substrate, waste from the slaughterhouse is used, resulting in high levels of ammonium and hydrogen sulphide. The ammonium can be recovered by extraction with the sulfuric acid obtained by desulfurization of the hydrogen sulphide. This process forms the valuable fertilizer ammonium sulphate.

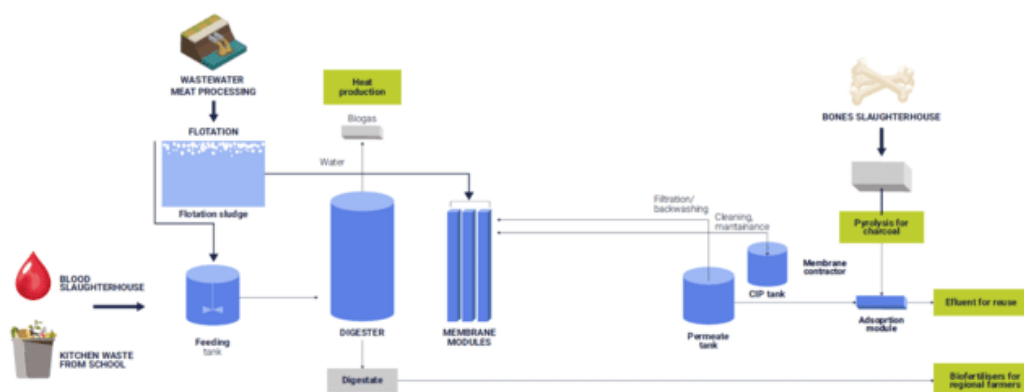


Figure 28. Austrian Ecosite: sources of waste, treatment processes and new added-value products

The new added-value products from the Austrian Ecosite are:

- Cleaned water for cooling or irrigation
- Biogas for energetic use
- Fertilizer for the surrounding farmers
- Char production

6.2 Description of the work (results obtained)

6.2.1 Char production

Solid waste streams, including bones, were processed and sent to the project partner Green for pyrolysis and characterization.

It was possible to pyrolyze the pork bones at 550°C and the characterization results are summarized in Table 60. Physic-chemical characterization of meat industry bone waste. The results of humidity, volatile at 550 °C, density and LHV of the pork bones as preliminary analysis are reported.

Table 60. Physic-chemical characterization of meat industry bone waste

Sample	Humidity (%)	Volatile at 550 °C (%)	Density(kg/m ³)	LHV (cal/g)
Pork bones	0.5	55	533	3426

From Table 60 it is observed that the pork bones wastes fit the pyrolysis requirements, regarding humidity and density, whereas the LHV is lower than 5,000 cal/g, this material does not fit the requirements to maintain the self-consumption of the plant (industrial point of view).

Volatile percentage is around 55 % this could indicate that low condensable gases (low liquid fraction) will be obtained in the pyrolysis test, so a prior with this material higher solid fraction will be obtained (char), further characterization is needed.

The following Table summarizes ion chromatography and analysis elemental results.

Table 61. Ion chromatography and elemental analysis results of meat industry waste

Sample	Fluorides (ppm)	Chlorides (ppm)	Bromides (ppm)	C (%)	H (%)	N (%)	S (%)	O (%)
Pork bones	111	193	<9.6	35.3	3.2	5.0	0.07	9.1

Results in Table 61 show that fluoride and chloride content in these samples are between 100-200 ppm, and bromide content is relatively low. Elemental analysis results show low carbon content around 35 %, and also low content for the rest of elements (H, N, O, S), in fact the sum of the percentage is ~ 53% (not 100) and remaining 47% is ashes.

Pork bones have also been analysed by ICP the results are summarized in Table 62.

Table 62. ICP analysis of sample pork bones in metal concentration (ppm).

Element	Analysis wavelength	Concentration [ppm]
Ag	328.068(A)	19.7
Al	396.152(A)	150
B	249.773(A)	181
Ba	233.527 (A)	3.16
Bi	223.061 (A)	95.1
Ca	317.933 (A)	250,740
Cd	214.438 (A)	<0.555
Co	228.616 (A)	0.562
Cr	267.716 (A)	5.2
Cu	324.754 (A)	14.5
Fe	259.941 (A)	42.9
Ga	417.206 (A)	<19.7
In	230.606 (A)	<40.5
K	766.491 (A)	1,682
Li	670.780 (A)	<0.602
Mg	280.270 (A)	3,268
Mn	257.611 (A)	0.462
Na	589.592 (A)	6,637
Ni	231.604 (A)	4.69
Pb	220.353 (A)	<19
Si	251.612 (A)	487
Sr	407.771 (A)	40.9
Tl	190.864 (A)	<3.51
Zn	213.856 (A)	91.7

The main component in pork bones, as was expected for bones, is calcium with 250,000.00 ppm sample also contains K, Mg, Si and Zn although in less proportion as was commented before this metal could hinder the development of porosity (activation process of char obtained).

Therefore, TGA curve analysis has also been performed. The data extracted from the curve is shown in Table 63.

Table 63. TG analysis of pork bones and the percentage of solid fraction at 550 °C.

Sample	Humidity (%)	Volatile (%)	Fixed Carbon (%)	Ash (%)	% at 550 °C
Pork bones	0.28	45.75	6.69	47.31	57

From TG analysis it is observed that at 550 °C, the remaining solid fraction is about 60%, however, it is confirmed that the ash percentage in this sample is high (47 %) and the fixed carbon is very low (7 %), within the solid fraction, 60 %, the 50 % are composed by ashes and only around 10 % is fixed carbon, so these characteristics are not adequate to obtain high yield of quality char.

Concluding the results show high ash content and moderate carbon yield. While technically feasible, optimization could improve char quality for activated carbon applications, but the original material appears to be not suitable for biochar production. A different utilization strategy has to be considered.

6.2.2 Biofertilizer production

Nutrient recovery is achieved through ammonia stripping and absorption processes. Hydrogen sulphide is removed from biogas using a desulphurisation column, producing sulphuric acid. This is used to recover ammonia via membrane contactors, forming ammonium sulphate. Reverse osmosis further concentrates nutrients, achieving high recovery efficiencies. The liquid ammonium sulphate solution can be either applied as a immediate fertilizer or can be mixed with the anaerobic digester sludge as a combined product. The ammonium from the ammonium sulfate solution is directly available for the plants, while the anaerobic sludge becomes slowly available for the plants and poses a long-term fertilizer and soil improver.

• High-value bio-fertilizer from hydrogen sulfate

The aim of the work package was to recover nutrients from streams generated by the anaerobic membrane bioreactor (AnMBR) and to produce a valuable fertilizer in the form of ammonium sulphate. Another objective was to reduce the burden of nitrogen in the form of ammonia from the permeate effluent and to clean the highly toxic and corrosive hydrogen sulphate from the biogas. Through the combination of different biological and membrane-based methods, these two problematic molecules are removed from the streams, transformed, and recovered as the targeted fertilizer. To achieve these goals, a biological desulphurization column (DSC) was built up and operated continuously subsequently to the AnMBR directly treating the produced biogas. For the recovery of ammonium, two different hollow fiber membrane modules (HFM) were tested. An in-house-crafted submerged module and a purchased conventional countercurrent module were tested with different ammonium concentrations to determine the transfer efficiency into the sulphuric acid solution. In addition, a reverse osmosis membrane and a nanofiltration membrane were tested for their retention capacity of ammonium at different pressure and pH settings. To further increase the biogas output, as well as the amount of ammonium and hydrogen Sulphur in the generated streams of the AnMBR, blood was deployed as a co-substrate in increasing amounts. Because of its high protein content, it contains a lot of Sulphur and nitrogen degraded to hydrogen sulphate and



ammonium, which poses problems in biogas production, but in this case is beneficial for fertilizer production.

Therefore, the desulphurization column was built up and installed on the laboratory scale AnMBR. The inoculation and operation were successful and the H_2S concentrations in the biogas have been substantially reduced. The pH of the H_2SO_4 solution reached a pH of 1.5, which corresponds to approximately $\sim 0.016 \text{ M H}_2\text{SO}_4$ solution.

The DSC demonstrated efficacy in reducing hydrogen sulphide (H_2S) in biogas. Initial H_2S concentrations of up to 2500 ppm were diminished to below 50 ppm post-treatment. The pH monitoring revealed that when the pH decreases to 1.5, the H_2S adsorption capability diminishes but recovers with the introduction of fresh media. Sulphate production coincided with H_2S removal, achieving concentrations of 2 g/L at a pH of 1.5. Although challenges with high H_2S input and process interruptions were identified, up to 100% H_2S removal was achieved during stable operations.

The submerged hollow fiber membrane modules were manufactured and tested at different concentrations. The results show the functionality of the membrane module and are proof of concept. The tests were repeated with a commercial countercurrent hollow fiber membrane module.

Submerged HFM assessed for ammonium absorption exhibited enhanced transfer at elevated ammonium concentrations. Transfer efficiency is associated with the shift in pH from NH_4^+ to NH_3 and the gradient of concentration across the membrane.

Countercurrent HFM experiments demonstrated that high initial ammonium concentrations and temperature enhance transfer rates, with a notable increase when pH and temperature rise in conjunction.

For water purification Reverse Osmosis and Nanofiltration were applied and the retention of ammonium and salts (conductivity) was tested.

RO was examined under varying pressures and ammonium concentrations, indicating nearly complete removal of Total Ammonium Nitrogen (TAN) and conductivity at low pH and high pressure. Elevated pressures led to increased membrane flow and retention, resulting in minimal ammonium in the permeate.

Analogous to RO, the performance of NF improved with reduced pH and augmented pressure. Although higher ammonium concentrations diminished the transmembrane flow, effective TAN removal remained feasible under optimized conditions.

Furthermore, the reverse-osmosis module (ROM) demonstrated high efficiency in ammonium recovery, achieving up to 98.6% retention at 18 bar pressure and a pH of 6, demonstrating the effectiveness of this method in concentrating ammonium in the retentate. These results underline the project's success in integrating biogas purification with nutrient recovery, yielding high-quality biogas and valuable ammonium sulphate, thus potentially providing both environmental and economic benefits in the future.

Overall, both RO and NF can achieve high removal efficiencies for TAN and conductivity, with neither offering a significant advantage over the other. Only the RO operation needs higher pressures to achieve the same results as the nanofiltration, resulting also in higher energy demands. It highlights the impact of operating parameters such as concentration, pressure, and pH on treatment efficacy.



- **Desulphuration column (DSC)**

The desulphurisation column was run continuously during the two co-digestion experiments with blood, resulting in higher H_2S levels in the AnMBR biogas stream. The medium was changed when the pH dropped to 1.5 corresponding to $\sim 0.016M$ H_2SO_4 (Figure 29).

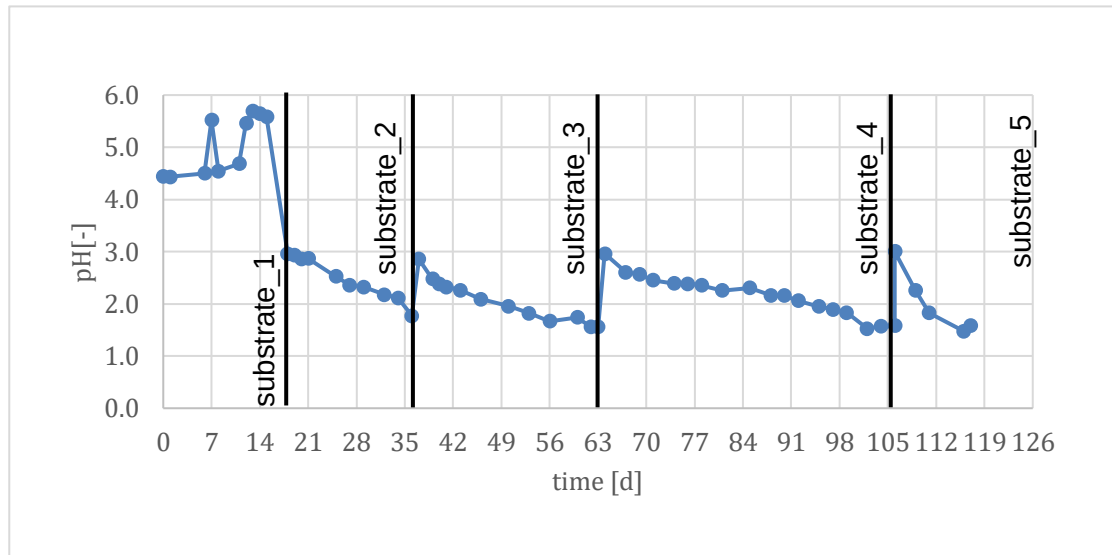


Figure 29: Development of the pH values in the first co-digestion experiment in the DSC substrate. Day of media exchange indicated by black vertical lines.

In the beginning, when the column was freshly inoculated, a substrate containing sulphur was used as an additive to supply the sulphur-oxidising bacteria with enough sulphur to oxidise until AnMBR produced higher levels (compare Figure 30).

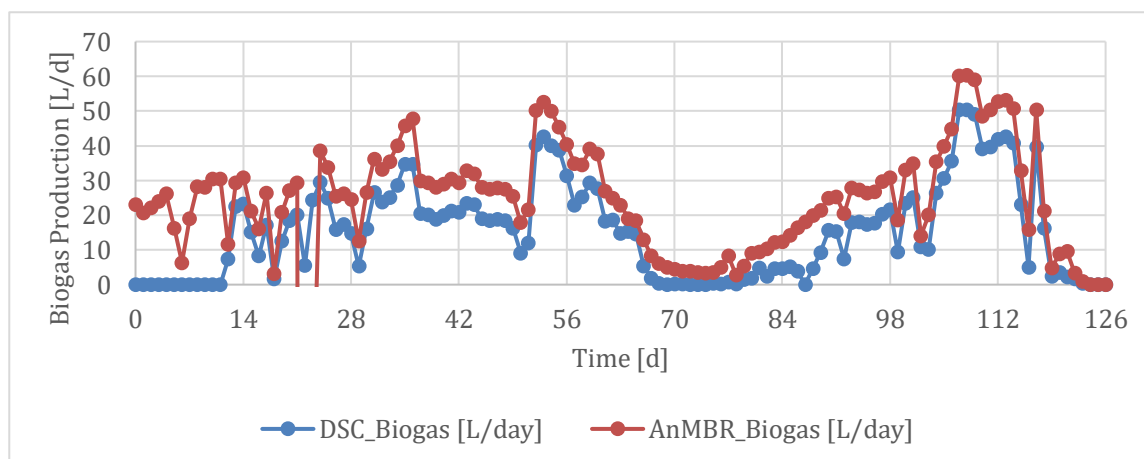


Figure 30: Amounts of biogas produced by the AnMBR and treated biogas by the DSC in Litre per day in the first co-digestion experiment.

When changing to fresh minimal mineral media, the pH started from pH 3 and dropped to about 1.5 corresponding to a $0.05M$ H_2SO_4 solution. Over the course of 126 days, the substrate had to be exchanged 4 times.

The gas quality before and after the Desulphurisation column (DSC) was analysed with an Awite device and the in and out flow H_2S concentration is illustrated in the following Figure 31. The H_2S

concentration in the AnMBR biogas reached levels of up to 2500ppm, but the DSC off-gas mostly showed levels below 50ppm. Once the pH of about 1.5 in the feeding solution was reached, the ability of the DSC for H₂S adsorption also decreased rapidly (peak 2., 3., 6.) but was fully recovered after fresh media was added. Also, high H₂S input and large biogas streams could lead to an overload of the DSC and higher levels of H₂S in the off-gas (peak 1., 4., 5.).

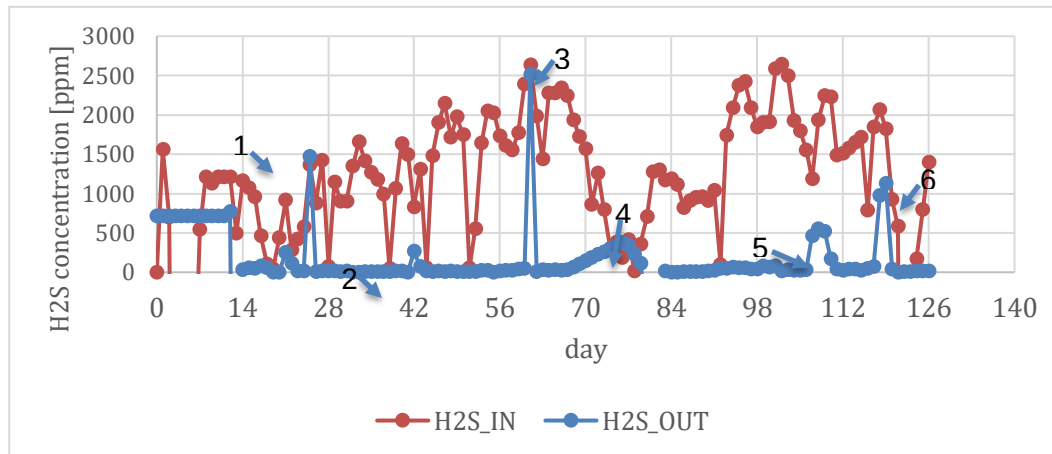


Figure 31: H₂S concentration in ppm in the biogas entering the DSC from the AnMBR (H₂S_IN) and the desulphurised biogas leaving the DSC (H₂S_OUT).

The amount of treated biogas was always lower compared to the biogas produced by the AnMBR, due to pressure loss in the tubing and the device, but also especially due to sampling for biogas quality measurements, which was about 3.5 to 7.5 litres per day, depending on the measurement frequency (12 or 24h).

The sulphate was measured with an Ion Chromatography and in Figure 32 the exponential increase of sulphate with the pH is illustrated for substrate 2, 3 and 4. The decrease in pH is correlated with the increase in sulphate, also depicted in Figure 33. The red dots resemble the measurements of substrate 2 and have been excluded for the calculation of the correlation. At a pH of 1.5 the sulphate concentration is about 2 g/L sulphate.

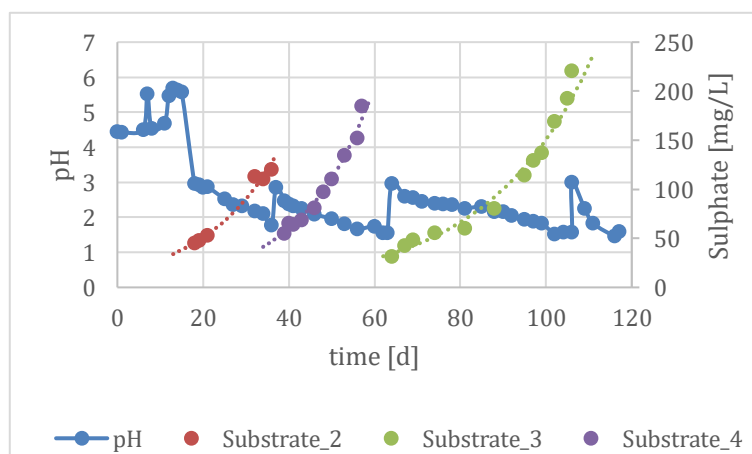


Figure 32. Development of the pH compared to the increase in sulphate [mg/L] concentration in the substrate over the course of 120 days in the first co-digestion experiment.

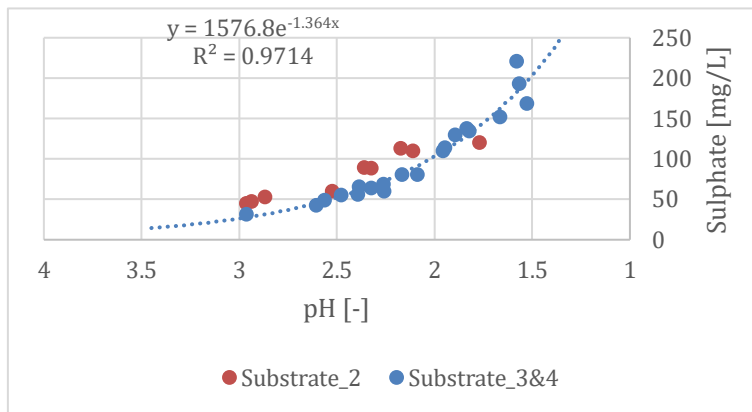


Figure 33. Correlation between the pH decrease and the increase in sulphate in the first co-digestion experiment. (Red dots from substrate_2 were excluded).

During the second experiment involving blood co-digestion, treatment was applied to up to 50 litres of biogas per day (Figure 34), resulting in elevated rates of hydrogen sulphide removal. The initial concentrations of H_2S in the biogas reached levels of up to 2000 parts per million (ppm), which were effectively reduced to mostly below 50 ppm after treatment.

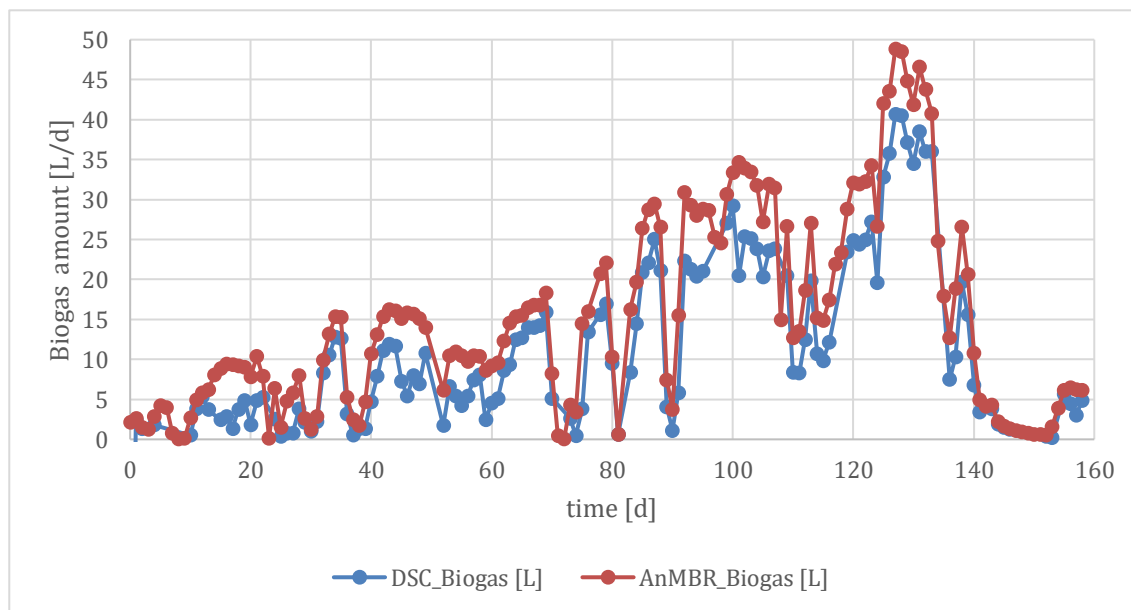


Figure 34: Biogas amount produced by the AnMBR in the second blood co-digestion experiment and corresponding treated biogas amounts from the DSC.

The hydrogen sulphate concentration reached up to 2000ppm and was mostly reduced to values below 50ppm, but in this period the DSC encountered multiple process problems, because of substrate pump failures, resulting in biogas streaming through the DSC untreated (40).

The reached removal rates were mostly between 80 and 100 % of H_2S removed from the initial AnMBR gas stream (Figure 35).

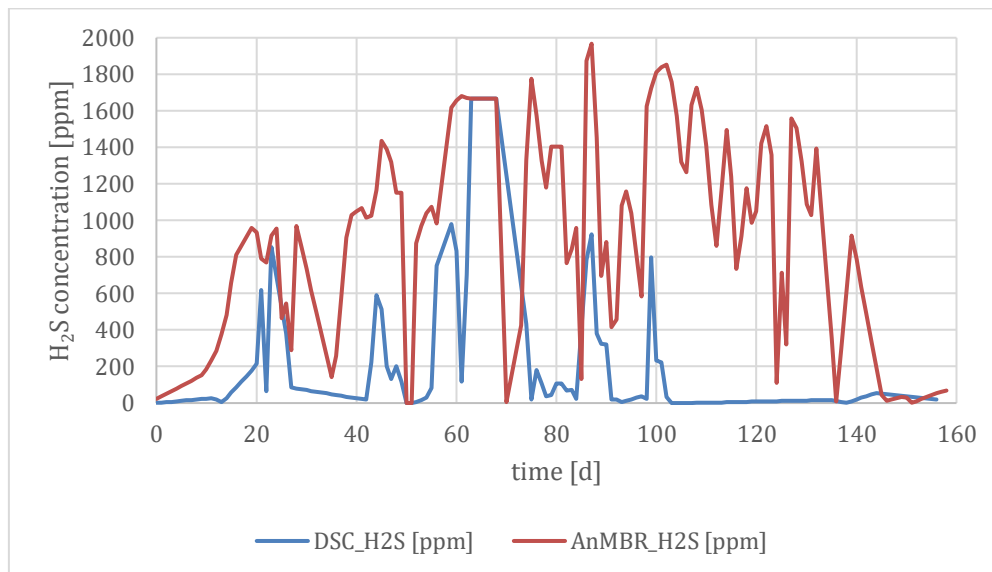


Figure 35. H_2S concentrations measured in the AnMBR biogas and DSC off gas in the second blood co-digestion experiment.

The removed hydrogen sulphide was converted into sulphate, achieving concentrations of up to 2.7 mg/L (Figure 36).

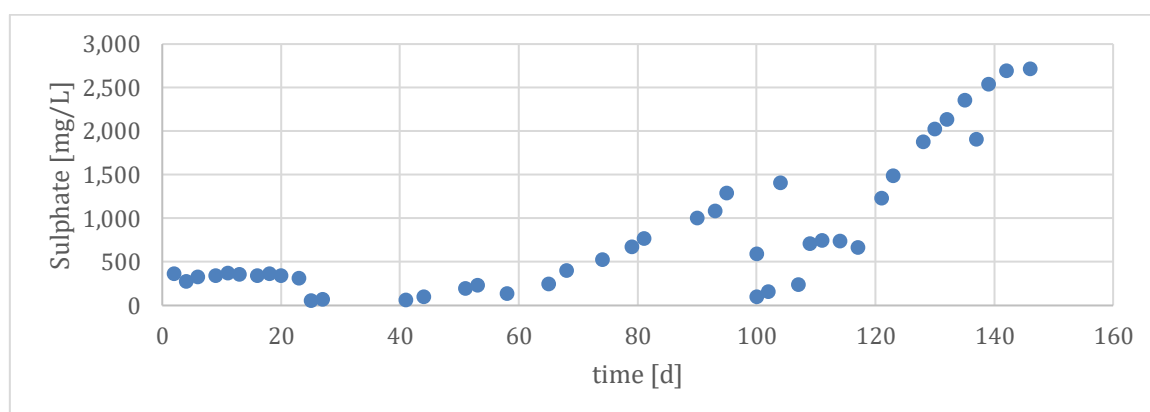


Figure 36. Sulphate concentration found in the DSC media over the course of the second blood co-digestion experiment.

Compared to the first blood co-digestion experiment, despite the problems higher sulphate concentrations were reached with lower H_2S concentrations but with similar biogas amounts.

- **Hollow fibre membrane modules (HFM)**

Submerged HFM

The performance of the hollow fibre membrane was tested by testing the absorption of ammonium in a 0.05M H_2SO_4 solution in 2.5 hours. Therefore, ammonium bicarbonate solutions in the concentration of 1, 3 and 6 g/L were prepared corresponding to 0.17, 0.49 and 1.10 g/L NH_4-N accordingly. The ammonium concentration in the sulphuric acid solution increased with time, depending on the initial starting concentration (Figure 37). With increasing ammonium concentrations, also the absorption increased. The effects were more pronounced with higher concentration of ammonium. The higher the concentration difference, the better the transfer coefficient.

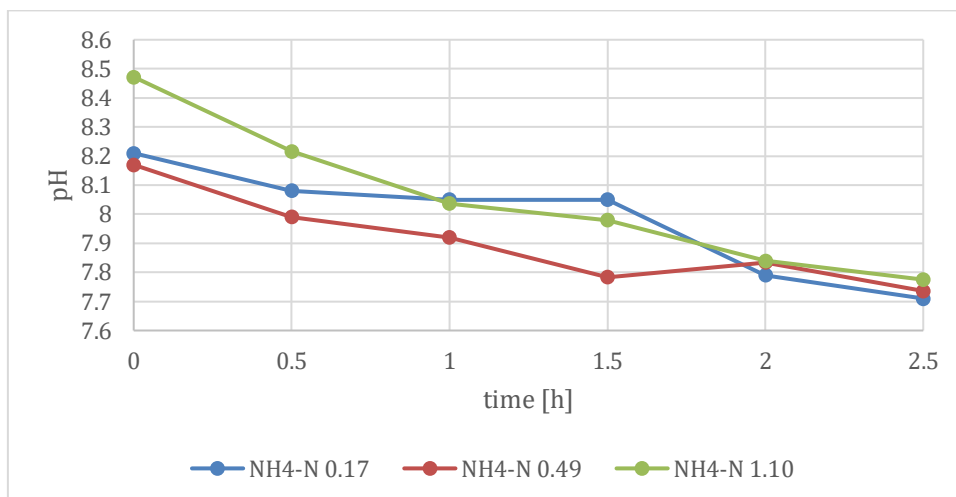


Figure 37. Development of the pH in the desorption ammonium decarbonate solutions over the course of 2.5 hours of experiment 1.

At the same time, the pH decreased in the ammonium bicarbonate solutions at about the same rate, also reaching comparable final pH values after 2.5 hours (**Figure 38**).

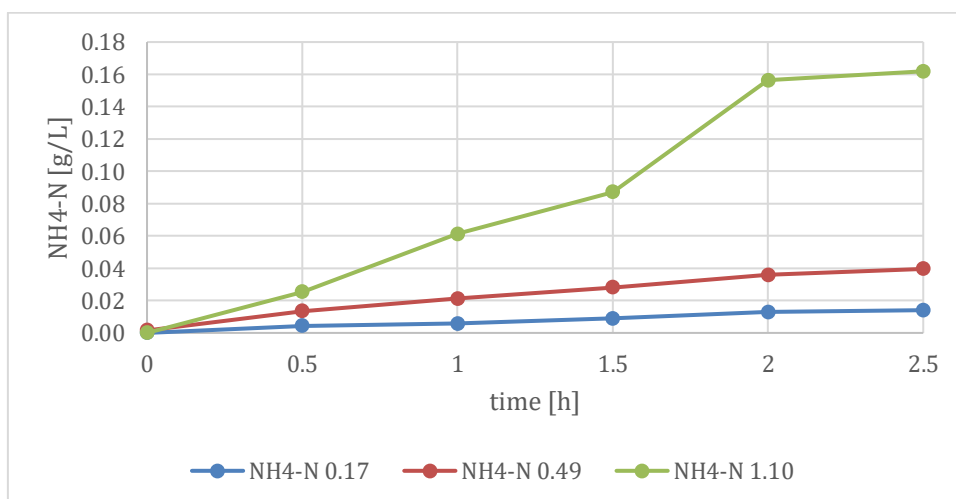


Figure 38. Accumulation of ammonium in the absorption solution 0.05M H₂SO₄ solution over 2.5 hours in the first HFM experiment

The transfer coefficient k of the ammonium from the ammonium bicarbonate solution into the sulphuric acid solution was similar between 0.17 and 1.10 g/L ammonium (**Figure 39**). The higher concentration of ammonium leads to a higher transmembrane potential increasing the transfer rate, but the higher pH also results in a shift from NH_4^+ to NH_3 passing through the membrane. This first experiment shows that higher concentrations are beneficial for higher transfer, but the pH plays an even greater role.

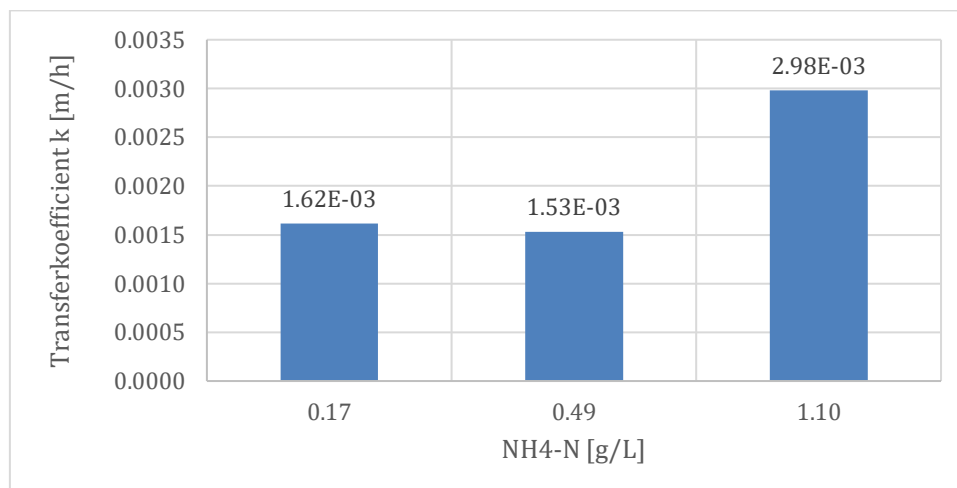


Figure 39. Transfer coefficient at different ammonium concentrations for the hollow fibre membrane experiment 1.

In a second experiment, higher concentrations were tested, namely ammonium concentrations of 1, 3 and 6 g/L (**Figure 40**). With increasing ammonium concentrations, the transfer was even more pronounced and at the highest starting concentration of 6 g/L ammonium was reached in the absorbing sulphuric acid solution after 2.5 h.

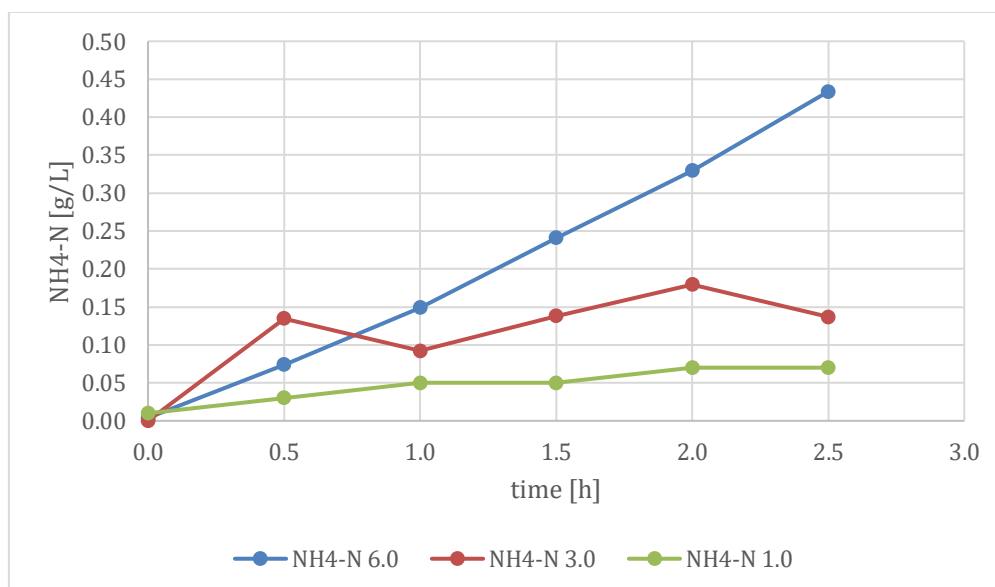
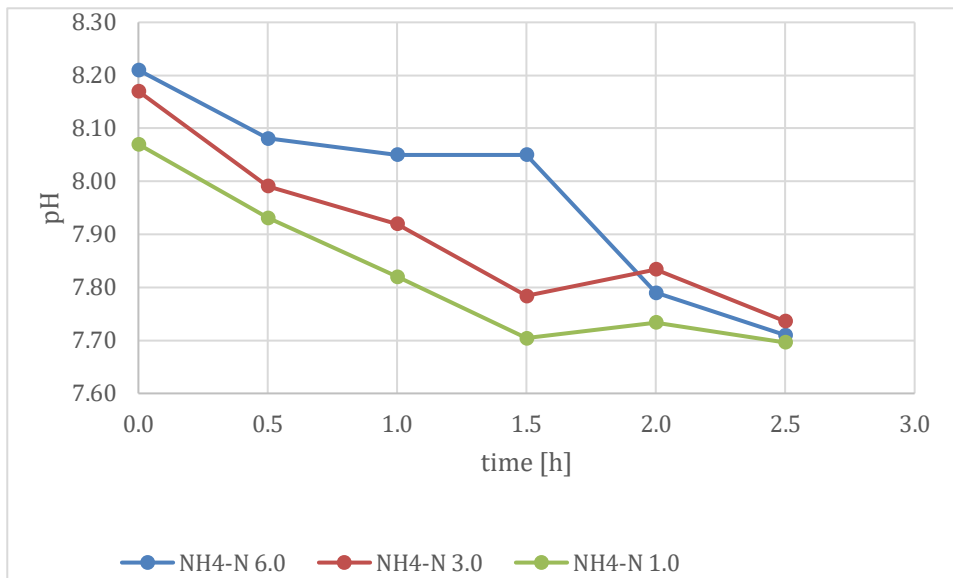


Figure 40. Accumulation of ammonium in the absorption solution 0.05M H₂SO₄ solution over 2.5 hours in the second experiment.

At the same time, the pH decreased in the ammonium bicarbonate solutions at about the same rate, also reaching comparable final pH values of 7.7 after 2.5 hours (Figure 47).



The transmembrane coefficient k increased with increasing starting concentration (**Figure 42**). At the lowest starting concentration, the transmembrane coefficient was 1.62×10^{-3} m/h. This increased at the highest starting concentration to 5.36×10^{-2} m/h. Thus, the transmembrane coefficient was 33 times higher at the highest concentration, compared to the lowest starting concentration. The relationship between the transfer coefficient and the initial ammonium concentration is seen to be exponential; therefore, a small increase in the concentration of $\text{NH}_4\text{-N}$ causes a very large increase in the diffusion rate. These results indicate an improvement in flow by concentration gradients.

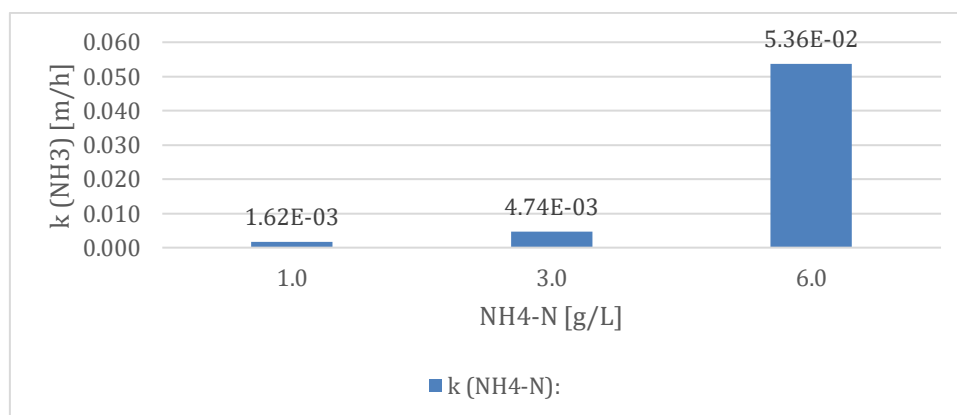


Figure 42. Transfer coefficient at different ammonium concentrations for the hollow fibre membrane experiment 2.

Countercurrent

In the counter current HFM experiment, ammonium nitrogen concentrations of 2, 4 and 6 g/L were tested. The absorbing sulphuric acid solution was cycled through the module lumen, whereas the ammonium solution was cycled through the hollow fibres. The ammonium concentration in both solutions was analysed in 30 min intervals over three hours. The results for the conditions of 20°C and pH 8 are shown in **Figure 43**.

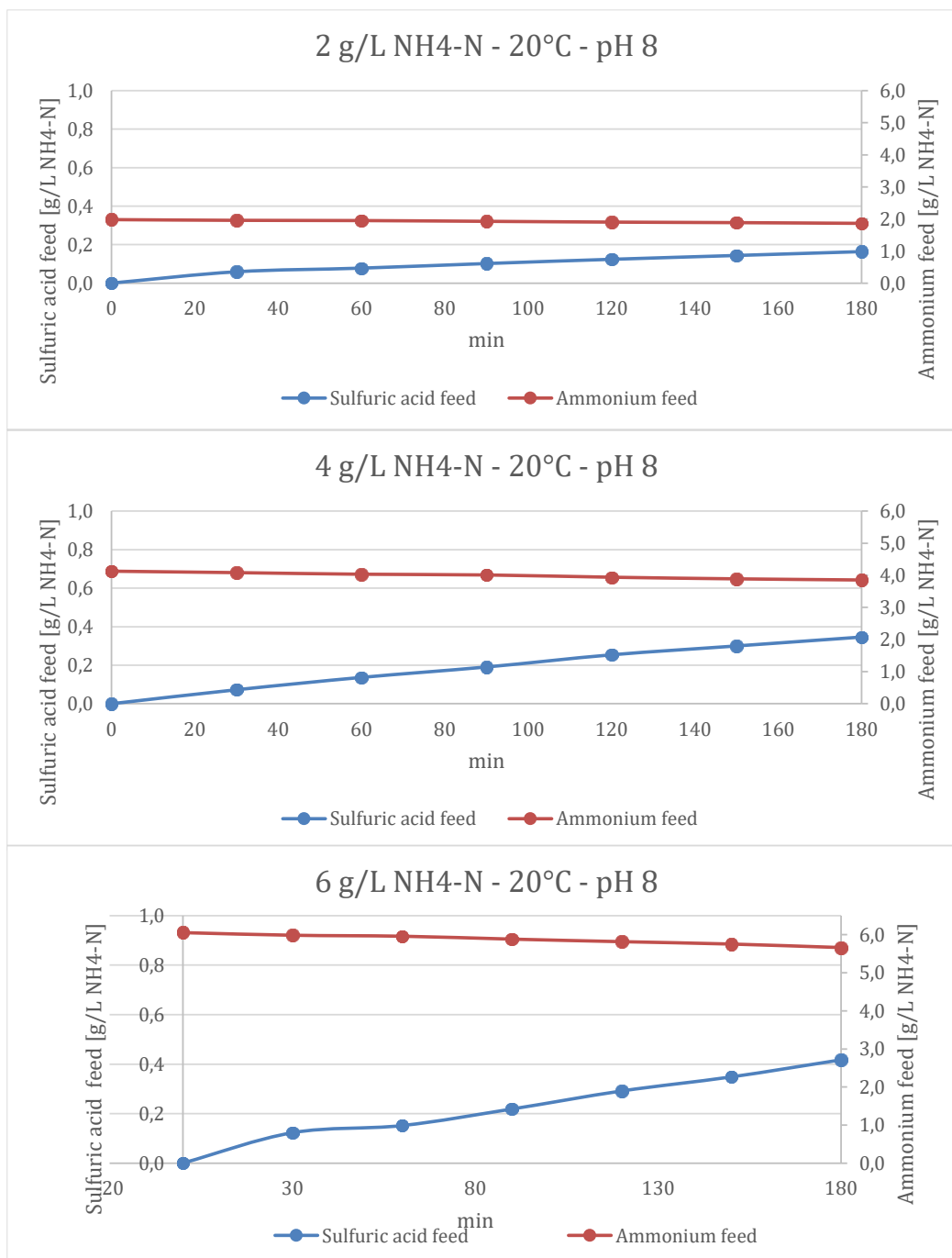


Figure 43. Countercurrent HFM experiment with 2,4 and 6 g/L Ammonium at 20°C and pH8.

In 180 min 0.16 g/L ammonium was reached with a starting solution of 2 g/L, 0.35 g/L with a starting solution of 4 g/L, and 0.42 g/L for the starting solution of 6 g/L. At all three concentrations, 7 to 10% of the initial concentration was reached in the sulphuric acid solution after 180 min. The higher the initial concentration, the higher also the achieved concentration in the sulphuric acid solution, but with the increase also the difference between the two solutions after 180 min increased. Therefore, for the recovery of nitrogen, high ammonium concentrations are better suitable, while for water recovery, low initial ammonium values are better resulting in a lower overall ammonium concentration. This can also be seen in the transfer coefficients which do not increase in the counter



current setup with increasing concentration. The speed of the transfer stays the same, but more ammoniums can be absorbed, because of higher concentrations.

Additional experiments were conducted to evaluate the influence of temperature and pH on ammonium transfer in the countercurrent HFM configuration. Tests performed at 30°C and 37°C and at pH values of 8 and 10 showed that increasing both temperature and pH significantly enhances ammonium transfer into the sulphuric acid solution. Under alkaline conditions (pH 10), nearly complete removal of ammonium from the feed solution was achieved within 180 minutes, while concentrations in the acid phase increased up to approximately 3.9 g/L. The results confirm that the transfer coefficient is strongly influenced by operational parameters. Higher temperatures and alkaline conditions promote the conversion of NH_4^+ to NH_3 , increasing diffusion through the membrane. The highest transfer rates were observed at 37°C and pH 10. Overall, the combination of high ammonium concentration, elevated temperature, and high pH resulted in optimal transfer performance, although transfer rates remained lower than those observed in the submerged HFM configuration.

- **Reverse-osmosis module (ROM)**

Another option besides the use of HFM for the recovery of nitrogen is the use of reverse osmosis or nanofiltration membranes. In a first experiment, the pilot reverse osmosis membrane module was tested with ammonium bicarbonate solutions at increasing concentrations starting from 0.5 g/L up to 6 g/L ammonium at pH 4.5, 6.0 and 7.5 and at 10, 14 and 18 bar.

The data compiled from the RO experiment with 0.5 g/L ammonium are presented in **Figure 44**. With the decrease in pH to 4.5 the flow across the membrane increased slightly and with the increase with the pressure substantially from 5 L/h at 10 bars up to 30 L/h at 18 bars. The feed conductivity was at all pH values about 25 mS/cm. In the permeate, the conductivity was reduced below 5 mS/cm and almost reached 0 at the higher-pressure setting. The conductivity removal achieved was almost 100% at pH 6 and 4.5 at 14 and 18 bars. The total ammonium nitrogen (TAN) removal showed similar results as the conductivity with TAN removal of almost 100% at pH 6 and 4.5 at 14 and 18 bars.

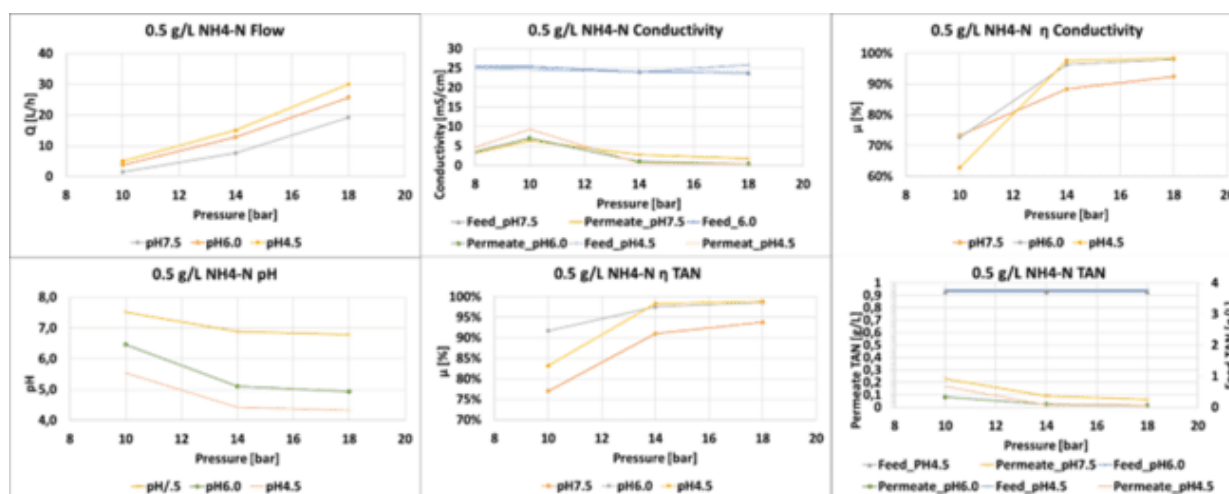


Figure 44. Compiled data of the reverse osmosis experiment with 0.5 g/L initial ammonium.

In **Figure 45** are presented the data collected from the 2 g/L ammonium RO experiment. The initial ammonium concentration of 2 g/L was held back and concentrated in the retentate. The maximum ammonium loss to the permeate was 0.2 g/L at 10 bar and pH 7.5. When the pressure was increased,



the lost ammonium was further decreased, but a decrease in pH was much more effective. The difference between pH 6 and 4.5 was only minor. Therefore, the addition of sulphuric acid to a pH of 6 was sufficient for high ammonium retention of 97% at 10 bar. With an increase in pressure to 18 bars, this increased even further to 98.6%.

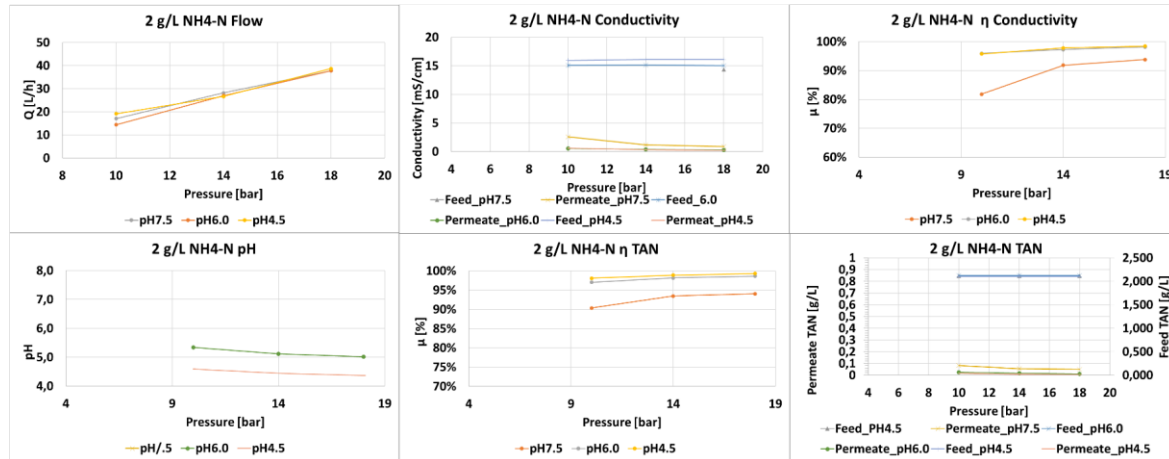


Figure 45. Compiled data of the reverse osmosis experiment with 2 g/L initial ammonium.

The data compiled from the 4 g/L ammonium RO experiment are shown in **Figure 46**. The same trends can be seen as in the two previous lower concentrations.

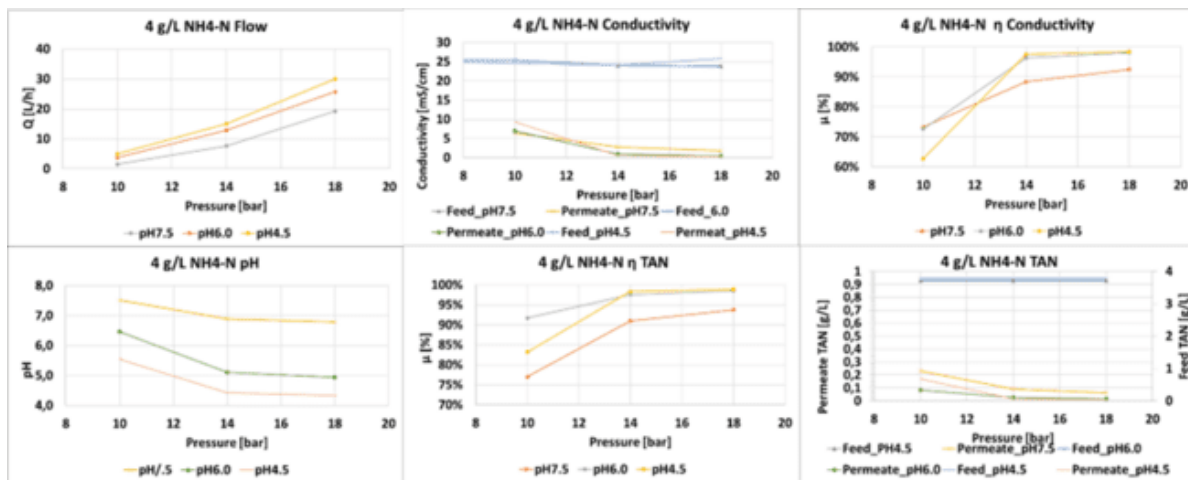


Figure 46. Compiled data of the reverse osmosis experiment with 4 g/L initial ammonium.

In **Figure 467** the compiled data of the RO experiment with 6 g/L ammonium is shown. At these concentrations, no flow at 10 bars was observed. Maximum transmembrane flow was 1000 L/h at 18 bars. At this high pressure, again removal rates of almost 100% for the conductivity and the TAN were reached at pH 6 and 4.5. At this very high initial ammonium concentration, the ammonium found in the RO permeate was below 0.1 g/L.

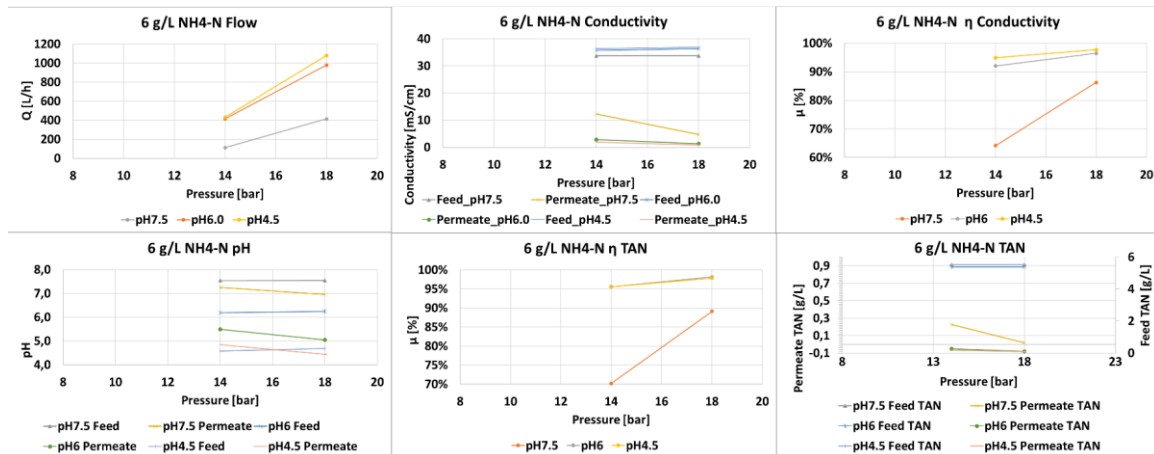


Figure 47. Compiled data of the reverse osmosis experiment with 6 g/L initial ammonium.

In conclusion from the results of the RO experiment, the membrane cross-flow velocity was not affected by the pH and only increased with pressure. Ammonium retention was reduced as pH levels rose and NH₄-N concentrations increased. However, it increased with increased pressure. In contrast, the concentration of ammonium in recovered water or reverse osmosis (RO) permeate increased with an increase in pH and NH₄-N levels but decreased with increased pressure. Overall, very high removal rates of conductivity and TAN were found, reaching almost 100 % at decreased pH levels and increased pressure of 18 bars.

The experiment was also conducted with permeate from the lab AnMBR displayed in Figure 48. The transmembrane flow was 25 L/h at maximum and reduced compared to the same ammonium concentration of 2 g/L ammonium bicarbonate. However, in addition to that, the removal of conductivity and TAN was as effective in the permeate as in the ammonium bicarbonate solutions, showing there is no matrix effect that diminishes the process.

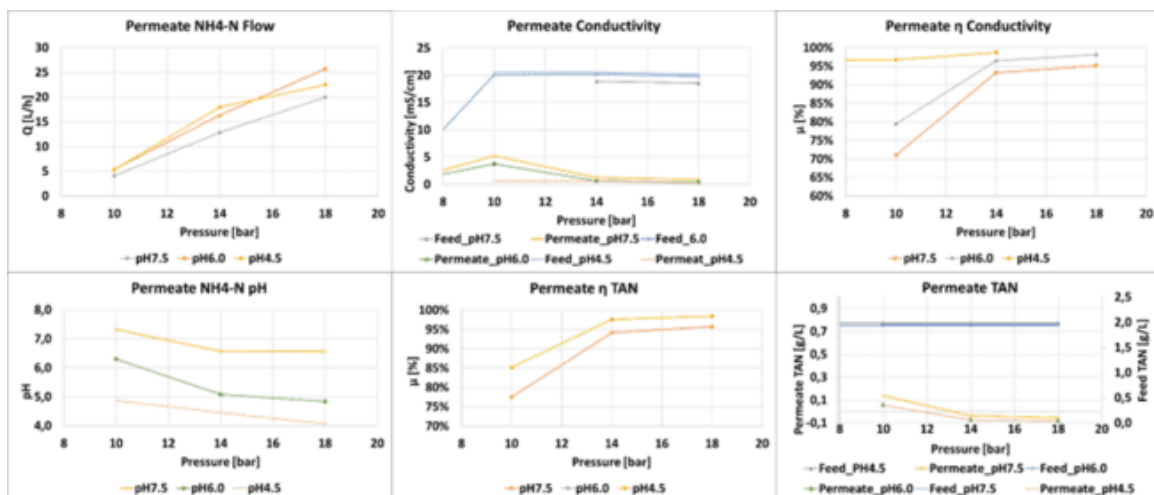


Figure 48. Compiled data of the reverse osmosis experiment with AnMBR permeate with a initial ammonium concentration of 2 g/L.

• Nanofiltration module (NF)

The experiments were repeated with the same initial ammonium concentrations of 0.5, 2, 4 and 6 g/L, but with a nanofiltration membrane instead. The pressure settings tested were 4, 6, 8 and 10

bars. The trans membrane flow conductivity measurements and conductivity removal effectivity are displayed in **Figure 49**.

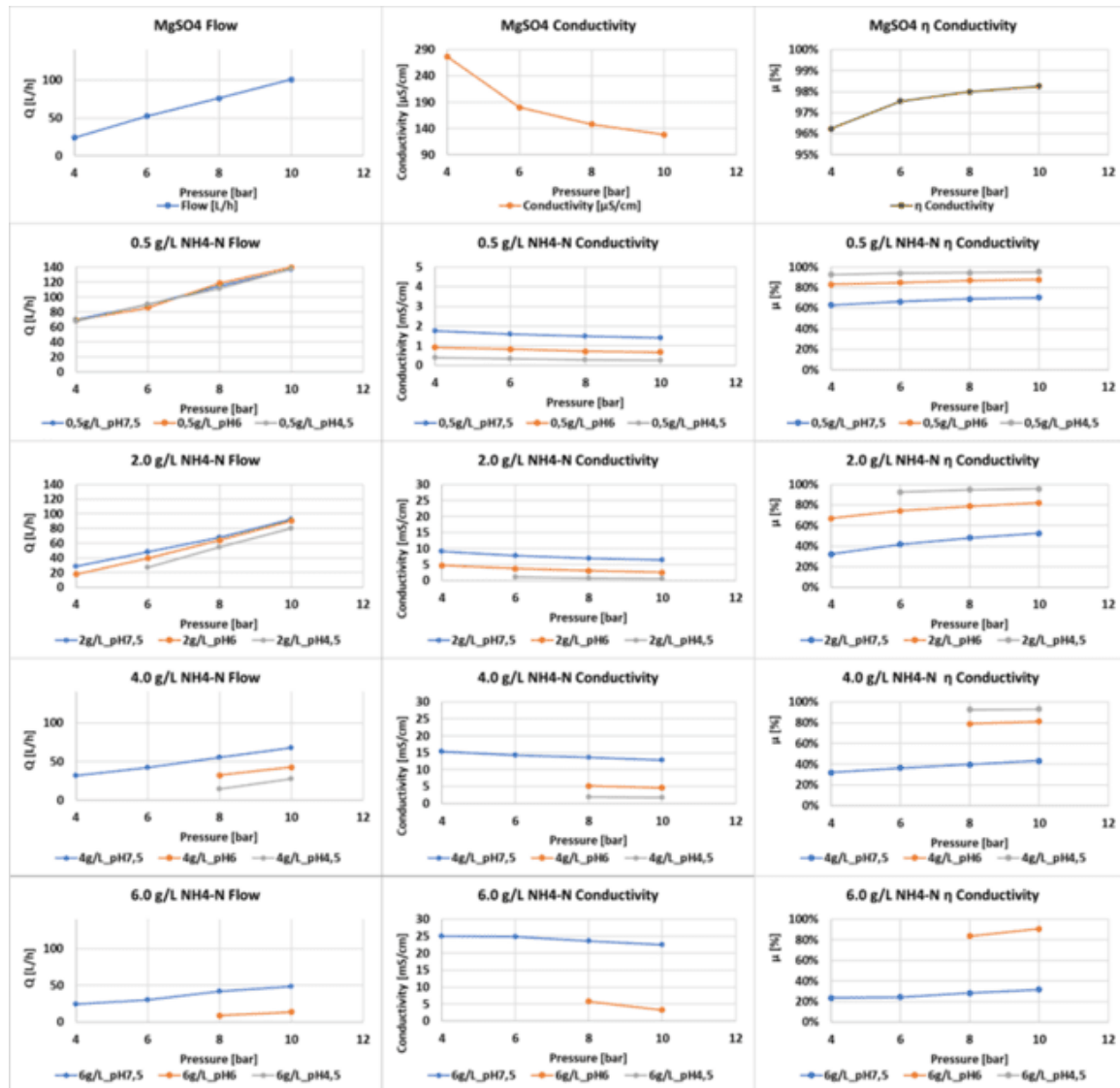


Figure 49. The trans membrane flow conductivity measurements and conductivity removal effectivity of the nanofiltration experiment with the initial ammonium concentrations of 0.5, 2, 4

The functionality of the nanofiltration membrane was first tested with $MgSO_4$. The flow increased with increasing pressure, and the conductivity removal increased to 98% with increasing pressure. The transmembrane flow increased with an increase in pressure but decreased with an increase in ammonium concentration. The maximum flow rate at 0.5 g/L TAN was 140 L/h and decreased to 50 L/h at 6 g/L TAN. The conductivity increased from 2 mS/cm at 0.5 g/L TAN to 25 mS/cm at 6 g/L TAN. The conductivity removal rate decreased with increasing ammonium concentration but increased at each concentration step with decreasing pH and increasing pressure. The highest removal rate for the highest concentration of 6 g/L was 90%, but higher removal rates were reached at the same settings for all other concentrations.

In **Figure 50** the TAN removal and ammonium concentrations of the experiment are depicted. With the decrease of the pH the TAN removal was increased at every concentration. And with increasing

pressure, removal was increased for each concentration and pH level. The highest removal rates, regardless of the initial concentration, were reached at a pH of 4.5 and a pressure of 10 bars. But the higher the initial ammonium concentration, the more it was also found in the NF permeate, even though almost 0 g/L was reached at 10 bars and a pH of 4.5.

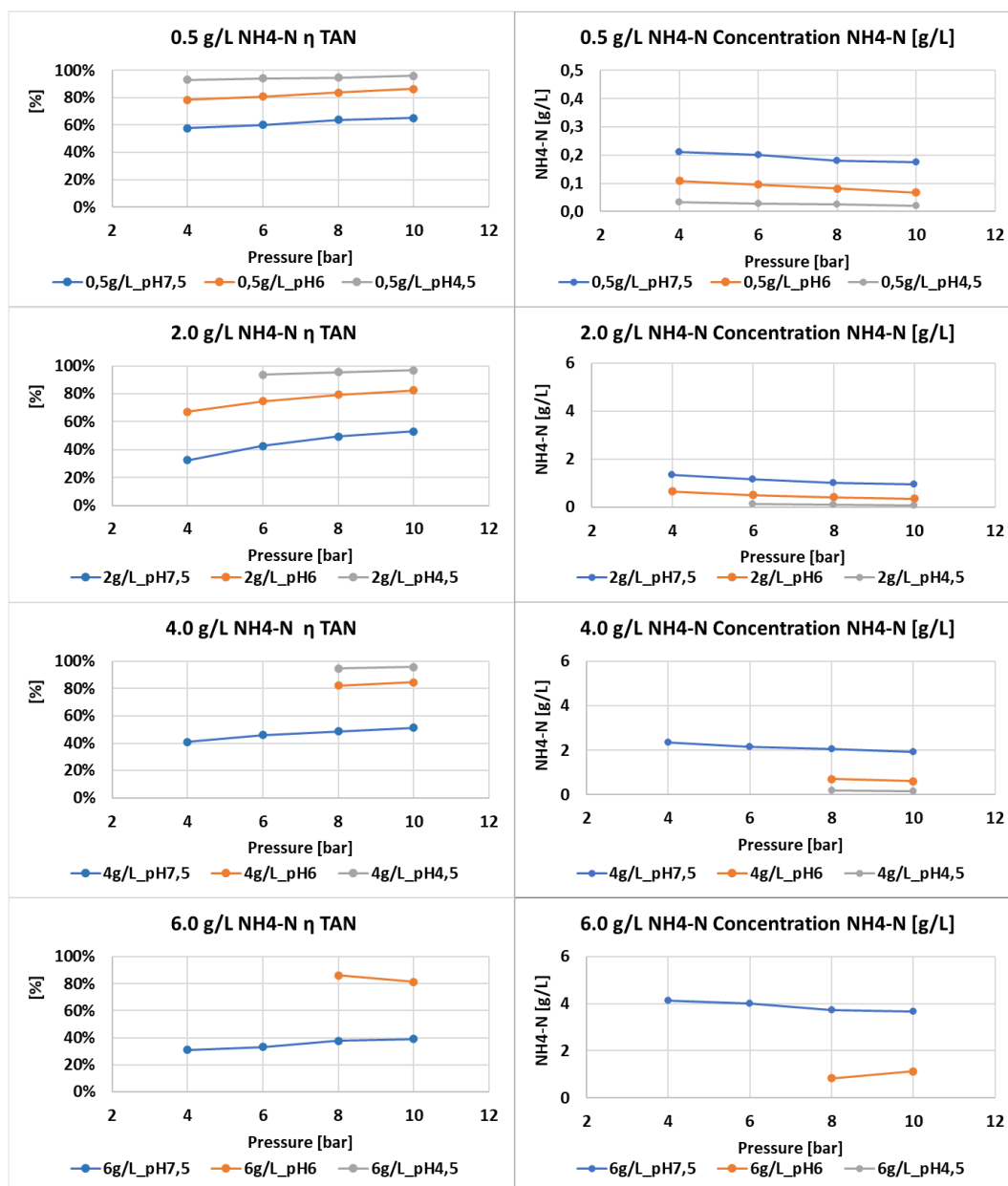


Figure 50. The TAN removal and ammonium concentration of the nanofiltration experiment with the initial ammonium concentrations of 0.5, 2, 4 and 6 g/L at 4, 6, 8 and 10 bars.

The RO membrane module and the nanofiltration module showed similar results and trends. In terms of ammonium and conductivity, neither the nanofiltration nor the reverse osmosis showed superior properties.

Conclusions

In the WP diverse methods for desulphurisation and ammonium recovery from biogas and wastewater streams were investigated.

The desulphurisation column was built up and installed on the laboratory scale AnMBR. The inoculation and operation were successful and the H_2S concentrations in the biogas have been substantially reduced. The pH of the H_2SO_4 solution reached a pH of 1.5, which corresponds to approximately $\sim 0.016 \text{ M H}_2\text{SO}_4$ solution.

The DSC demonstrated efficacy in reducing hydrogen sulphide (H_2S) in the biogas. Initial H_2S concentrations of up to 2500 ppm were diminished to below 50 ppm post-treatment. The pH monitoring revealed that when the pH decreases to 1.5, the H_2S adsorption capability diminishes but recovers with the introduction of fresh media. Sulphate production coincided with H_2S removal, achieving concentrations of 2 g/L at a pH of 1.5. Although challenges with high H_2S input and process interruptions were identified, up to 100% H_2S removal was achieved during stable operations.

The submerged hollow fibre membrane modules were manufactured and tested at different concentrations. The results show the functionality of the membrane module and are a proof of concept. The tests were repeated with a commercial countercurrent hollow fibre membrane module. Submerged HFM assessed for ammonium absorption exhibited enhanced transfer at elevated ammonium concentrations. Transfer efficiency is associated with the shift in pH from NH_4^+ to NH_3 and the gradient of concentration across the membrane.

Countercurrent HFM experiments demonstrated that high initial ammonium concentrations and temperature enhance transfer rates, with a notable increase when pH and temperature rise in conjunction.

In the WP notable results in improving biogas quality and producing valuable by-products through innovative purification and recovery techniques were archived. The desulphurisation column (DSC) consistently removed hydrogen sulphide (H_2S) from the biogas stream. The process also produced sulphuric acid (H_2SO_4), which can be further used in a next step in the hollow fibre membrane modules (HFM) to absorb ammonia (NH_3) from the permeate stream. The HFM experiments showed that higher ammonium concentrations and lower pH levels enhanced the transfer efficiency, with the highest transfer coefficient observed at 6 g/L ammonium bicarbonate solution.

For water purification Reverse Osmosis and Nanofiltration were applied and the retention of ammonium and salts (conductivity) was tested.

RO was examined under varying pressures and ammonium concentrations, indicating nearly complete removal of Total Ammonium Nitrogen (TAN) and conductivity at low pH and high pressure. Elevated pressures led to increased membrane flow and retention, resulting in minimal ammonium in the permeate.

Analogous to RO, the performance of NF improved with reduced pH and augmented pressure. Although higher ammonium concentrations diminished the transmembrane flow, effective TAN removal remained feasible under optimised conditions.

Furthermore, the reverse-osmosis module (ROM) demonstrated high efficiency in ammonium recovery, achieving up to 98.6% retention at 18 bar pressure and a pH of 6, demonstrating the effectiveness of this method in concentrating ammonium in the retentate. These results underline the project's success in integrating biogas purification with nutrient recovery, yielding high-quality



biogas and valuable ammonium sulphate, thus potentially providing both environmental and economic benefits in the future.

Overall, both RO and NF can achieve high removal efficiencies for TAN and conductivity, with neither offering a significant advantage over the other. Only the RO operation needs higher pressures to achieve the same results as the nanofiltration, resulting also in higher energy demands. It highlights the impact of operating parameters such as concentration, pressure, and pH on treatment efficacy.

- **Anaerobic sludge for fertilizer**

The data of the analytics and production of the anaerobic digestate is already represented in detail in the deliverable D4.3.

The anaerobic digester is build with a pneumatic mixing system composed of an inner chamber and an outer chamber connected by a valve. Pressure is built up by the production of biogas in the outer chamber, while the inner chamber is connected to the gas storage and no pressure can build up. When fresh substrate is applied the valve is opened and the pressure is released thereby facilitating the mixing of the fresh substrate into the slurry. This is important so microorganisms get in contact with the fresh substrate and can metabolize it to biogas. To assess the mixing quality and the best spot for digestate withdrawal as fertilizer the anaerobic reactor was sampled at three different outlets. On the top (OUT; inner chamber), where the digestate overflow is located releasing anaerobic sludge every time new substrate is applied, the middle (DIG; outer chamber) and the bottom (Sludge; both chambers). The analytical data and the sampling spots are represented in **Figure 51**.

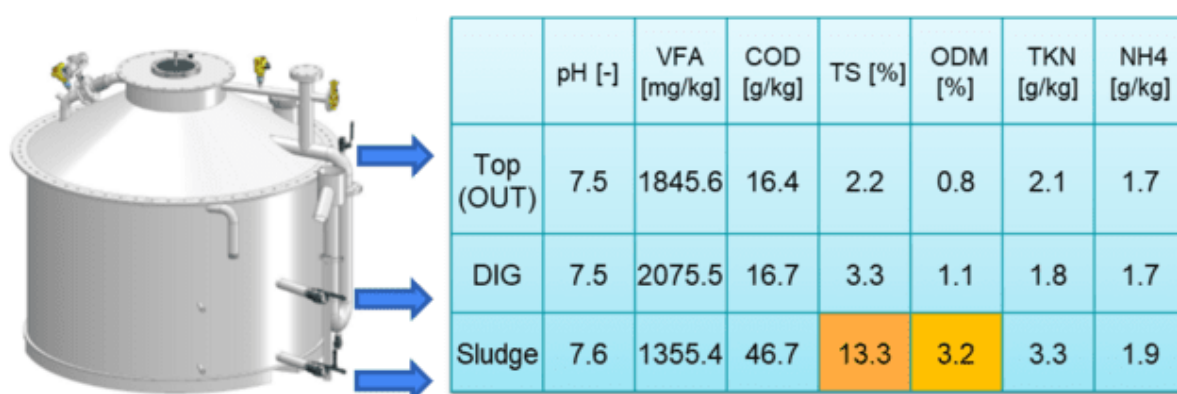


Figure 51. Analytical data from the different sampling spots in the anaerobic digester.

The data shows high variations in the solids and volatile solids content from the top to the bottom. The top and middle do not differentiate as highly as to the bottom values. In the bottom fraction the total solid content is 13.3% and a very low organic content of 3.2%. This indicates the accumulation of inorganic substances, especially iron from the flocculation sludge used as substrate. Even though the ratio of total to organic solids is better, the overall organic fraction is less with 2.2% and 0.8% respectfully. Especially the high phosphor in the bottom fraction makes it more suitable for a high valuable fertilizer. The best option would be to mix the top and bottom fraction to adapt the fertilizer to the needed share of organics, phosphor and nitrogen.

The biogas plant currently produces 400 L/day of anaerobic digestate, a valuable fertiliser that could be distributed by cooperative farmers supplying livestock to Berger for meat production. The organisation of farmers into a cooperative facilitates logistical coordination and distribution. Additionally, these farmers can also contribute their pig manure to the biogas plant. Anaerobic digestion of pig manure is a well-established process that is considered a refinement, as the



application of anaerobic digestate to agricultural fields presents numerous advantages over pig manure. Anaerobic digestate offers superior availability of nutrients, particularly in terms of nitrogen present in ammonium². It also shows a significant reduction in pathogens compared to pig manure³ and mitigates the emissions of odorous compounds, methane, and ammonia (Amon et al., 2006). Furthermore, digestate application fosters long-term improvement of soil structure and promotes microbial activity, thus contributing to sustainable soil management⁴. The enhancement of this digestate fertiliser could be achieved through the production of ammonium sulphate from hydrogen sulphate found in biogas, as well as through the recovery of ammonium from permeate.

In Austria, the application of biogas digestate as fertiliser is governed by the Fertiliser Act 2021 (Düngemittelgesetz 2021) and the Fertiliser Ordinance 2004 (Düngemittelverordnung 2004, BGBl. II Nr. 100/2004). These regulations stipulate the permissible nutrient content and quality standards for fertilisers, including those derived from biogas digestate. The regulations include provisions on nutrient content limits, heavy metal thresholds and application restrictions. Compliance with these regulations is mandatory for all entities involved in the production, distribution, and application of biogas digestate as fertiliser. Regular monitoring and reporting are also required.

The digestate discharged from the top and the bottom of the reactor was assessed with respect to its suitability for use as a biofertilizer. To this end, additional comprehensive analyses were performed to determine its elemental composition of the bottom fraction since its high share of inorganics poses the higher threat in metal ion and heavy metal contents (see **Table 64**). The nitrogen content exceeds the minimum threshold of 0.2% N required for fertilizer application; however, the organic fraction of the total solids should constitute at least 50%. This criterion is only marginally satisfied by the investigated digestate.

² Möller, K., & Müller, T. (2012). "Effects of anaerobic digestion on digestate nutrient availability and crop growth: A review." *Engineering in Life Sciences*, 12(3), 242–257.

³ Arthurson, V. (2009). "Closing the global energy and nutrient cycles through application of biogas digestate to agricultural land – Potential benefits and drawbacks." *Energies*, 2(2), 226–242.

⁴ Alburquerque, J. A., de la Fuente, C., & Bernal, M. P. (2012). "Chemical properties of anaerobic digestates affecting C and N dynamics in amended soils." *Agriculture, Ecosystems & Environment*, 160, 15–22.



Table 64. Elemental composition of the digester sludge in accordance with Düngemittelverordnung 2004

Element	Fresh matter	Dry matter	Limits according to DMVO(2004)
TKN	3380 mg/kg FM	12472.32 mg/kg TS	-
NH ₄ -N	2090 mg/kg FM	7712.18 mg/kg TS	-
Al	838 mg/kg FM	3100 mg/kg TS	-
B	<0.19 mg/kg FM	<0.71 mg/kg TS	-
Ca	3460 mg/kg FM	12800 mg/kg TS	-
Co	0.76 mg/kg FM	2.8 mg/kg TS	-
Cr	11.1 mg/kg FM	41 mg/kg TS	100 mg/kg
Cu	<0.30 mg/kg FM	<1.10 mg/kg TS	-
Fe	49100 mg/kg FM	181000 mg/kg TS	-
Mg	944 mg/kg FM	3490 mg/kg TS	-
Mn	214 mg/kg FM	789 mg/kg TS	-
Mo	<0.03 mg/kg FM	<0.11 mg/kg TS	-
Na	1020 mg/kg FM	3770 mg/kg TS	-
Ni	1.81 mg/kg FM	6.69 mg/kg TS	100 mg/kg
P 4,66% P ₂ O ₅	20300 mg/kg FM	75100 mg/kg TS	-
S	978 mg/kg FM	3610 mg/kg TS	-
Se	<0.104 mg/kg FM	<0.384 mg/kg TS	-
Zn	31.3 mg/kg FM	116 mg/kg TS	-
K 0.09% K ₂ O	746 mg/kg FM	2760 mg/kg TS	-
As		<0.001 mg/kg TS	40 mg/kg
Cd		<0.005 mg/kg TS	3 mg/kg
Pb		<0.0005 mg/kg TS	100 mg/kg
Hg		<0.0002 mg/kg TS	1 mg/kg

The iron concentration is very high, although iron is not explicitly regulated in the Austrian fertilizer legislation. Optimization and reduction of the iron (III) chloride dosage applied during the flotation process could improve the TS/ODM ratio and thereby enable compliance with the relevant requirements. The potassium content of the sludge is below the regulatory threshold for fertilizers, which is 0.3% of the fresh mass, whereas only 0.09% was achieved. Consequently, potassium would either need to be supplemented or its input into the process increased via the feedstock.

In contrast, the phosphorus content is highly advantageous, corresponding to 4.6% P₂O₅ equivalents, while only 0.1% is required. The elevated phosphorus concentration originates from the wastewater, where it is enriched in the flotation sludge subsequently fed to the anaerobic digester. Phosphorus does not accumulate in the digester to a significant extent but is only partially assimilated by the microbial biomass.

Potential application

The potential application concept for the Austrian EcoSite is illustrated in **Figure 52**.

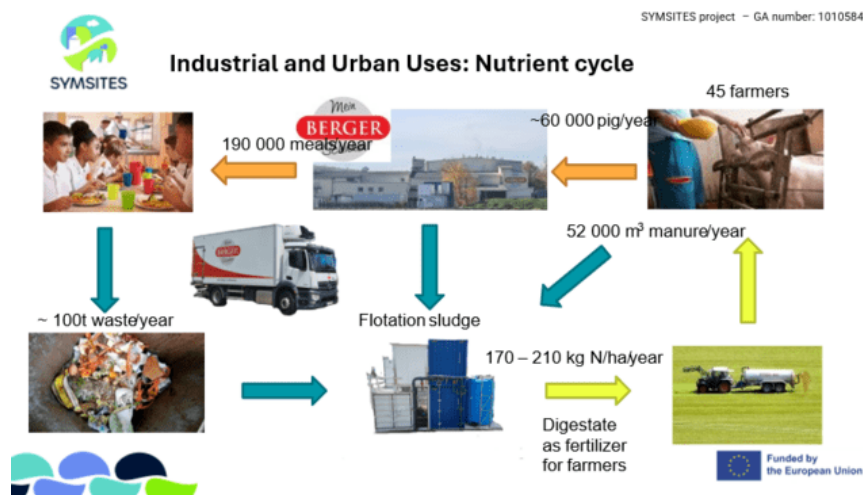


Figure 52. Austrian EcoSite nutrient circle example.

The company Berger provides 190,000 meals per year to surrounding schools and kindergartens. The food waste could be collected without any logistical effort upon meal delivery. The collected food waste potential is 100 tons per year which can be used for biogas production. The resulting hydrogen sulphate in the biogas can be recovered as sulfuric acid in a biological desulfurization column. The resulting sulfuric acid can be further used to recover the ammonium contained in the reclaimed water. In combination with the anaerobic sludge this would be a valuable biofertilizer for the surrounding 45 farmers supplying about 60,000 pigs per year to the company. Additionally, the farmers could bring about 52,000 m³ of pig manure per year to the biogas plant to get converted into energy and fertilizer saving energy for the intensive Haber-Bosch process in the fertilizer production. And also keeping the nutrients in a local loop.

6.2.3 Biogas for energetic use

The biogas produced by the anaerobic digestion is because of the high protein content in the biowaste and slaughterhouse waste rich in protein which is also degraded to hydrogen sulfate. The hydrogen sulfate is converted to sulfuric acid for fertilizer production in the desulfurization column and thereby additionally upgrading the biogas. The biogas has to be cleaned from the hydrogen sulfate before further use due to its highly corrosive and toxic character. The cleaned biogas can directly be used for electricity production in a gas turbine. In the process 1 m³ of methane is converted in to 10 kWh of electric. The same amount is lost in heat energy which can be recovered for heating of households or hot water supply. The electricity is directly used by the company for their high electricity demand of the cooling systems, while the heating can be supplied to a local distant heating network.

The methane yield of an anaerobic digester is strongly influenced by the type of substrate employed. The methane potential was tested for different substrates illustrated in **Figure 53**. Flotation sludge, which is characterized by a high lipid content, yields approximately 8.6 Nm³ CH₄ per ton of fresh matter (FM). Although lipids exhibit a higher specific methane potential than carbohydrates and proteins, the flotation sludge investigated contains only about 3 % total solids in the fresh matter. Consequently, the methane yield per ton of FM from ham and blood is substantially higher, at 96.8 and 84.6 Nm³/t(FM), respectively. But these protein rich feedstocks also pose problems if applied too much, because of their high nitrogen content which could lead to inhibition of the microbial

community. Therefore, a mixture of organic waste, slaughterhouse waste (blood) and flotation sludge lead to the most stable process and high methane yields.

Methane potential flotation sludge	Results
$Y(\text{CH}_4)$ in $\text{Nm}^3/\text{t}(\text{FM})$	8.6
$Y(\text{CH}_4)$ in $\text{Nm}^3/\text{t}(\text{COD})$	329.5
$Y(\text{CH}_4)$ in $\text{Nm}^3/\text{t}(\text{VS})$	498.1

Methane potential fermenter sludge	Results
$Y(\text{CH}_4)$ in $\text{Nm}^3/\text{t}(\text{FM})$	1.5
$Y(\text{CH}_4)$ in $\text{Nm}^3/\text{t}(\text{COD})$	478.6
$Y(\text{CH}_4)$ in $\text{Nm}^3/\text{t}(\text{VS})$	1066.6

Methane potential ham	Results
$Y(\text{CH}_4)$ in $\text{Nm}^3/\text{t}(\text{FM})$	96.8
$Y(\text{CH}_4)$ in $\text{Nm}^3/\text{t}(\text{COD})$	355.5
$Y(\text{CH}_4)$ in $\text{Nm}^3/\text{t}(\text{VS})$	464.4

Methane potential blood	Results
$Y(\text{CH}_4)$ in $\text{Nm}^3/\text{t}(\text{FM})$	84.6
$Y(\text{CH}_4)$ in $\text{Nm}^3/\text{t}(\text{COD})$	170.0
$Y(\text{CH}_4)$ in $\text{Nm}^3/\text{t}(\text{VS})$	473.0

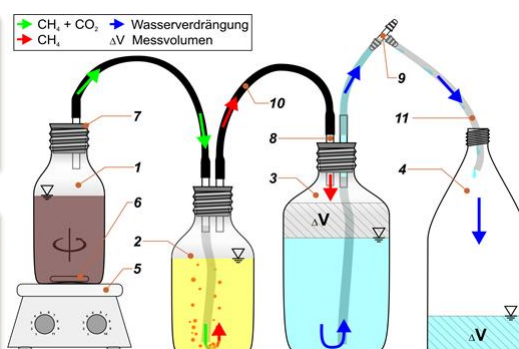


Figure 53. Biogaspotential test results for different substrates. Batchtest setup on the right with 1=Inoculum and substrate flask; 2=NaOH solution flask for CO_2 absorption; 3=Water flask for methane volume measurement; 4= Flask to collect the water volume equal

Based on the input quantities, the chemical composition of the substrate, and the amounts and composition of the biogas, analyses were carried out to determine the biogas yield per unit of fresh material (FM). Furthermore, the normalised methane yield was calculated based on the applied chemical oxygen demand (COD) and volatile total solids (VTS), with the results presented in **Figure 54**. The raw biogas yield was significantly the highest for wastewater and blood feed. However, when considering the yield of methane per chemical oxygen demand (COD), the results were comparable to those of other feed types. Specifically, with wastewater alone at a Hydraulic Retention Time (HRT) of 70 days, the raw biogas yield was the lowest, yet the methane yield per COD was markedly the highest. This phenomenon can be attributed to the fact that not all biomolecules from the wastewater and blood feed (WW + BL) were degraded to biogas and therefore accumulated in the reactor, only to be converted during the subsequent wastewater feed (WW).

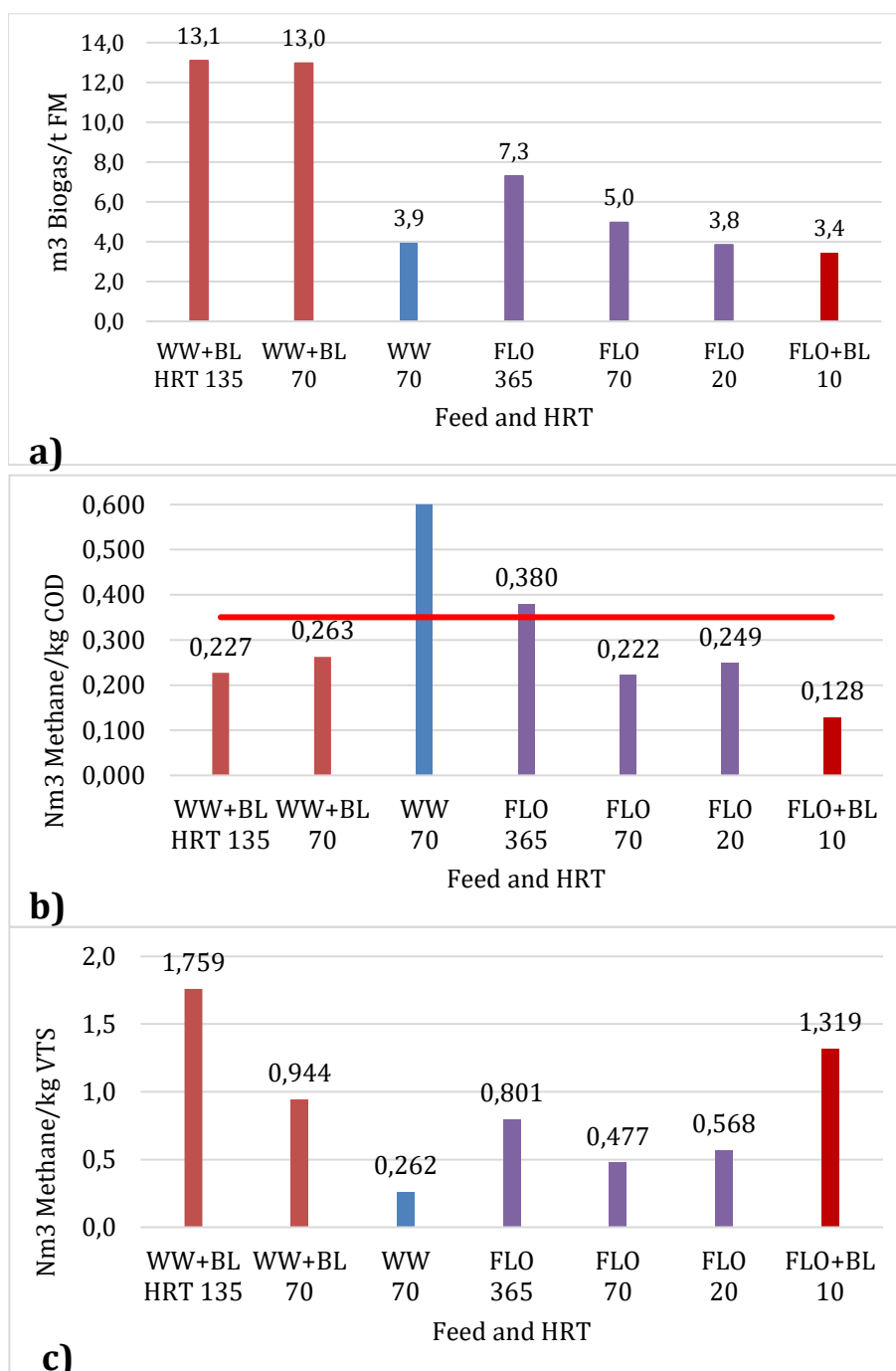


Figure 54. a) Biogas yield per fresh material. b) Methane yield per applied COD and theoretical maximum displayed as red horizontal line. c) Methane yield per applied VTS.

Furthermore, with a decrease in HRT, the raw biogas yield per unit of fresh flotation concentrate material decreased. A similar trend was observed with the methane yield per COD or volatile total solids (VTS). The simultaneous increase in feed and the reduction in HRT did not allow the microbial community responsible for methanogenesis to reach the cell density necessary for effective and rapid degradation of the substrate to methane. The lowest yields for biogas per fresh material (FM) and methane per COD were observed in the flotation concentrate and blood (FLO+BL) feed. This was due to the increase in the amount of feed and low HRT coinciding with the introduction of the

blood co-substrate, which led to an overload of the Anaerobic Digestion (AD) system. However, with increasing microbial cell densities and adaptation to the new co-substrate, the methane yield increased. In particular, the most conclusive parameter the methane yield per unit of applied volatile total solids presented different results: for the WW+BL feed, the yield initially decreased, reaching its lowest point with the WW feed, while achieving a notably high yield of 1.3 with the FLO+BL feed.

Based on the results from the pilot plant operation an energy scenario presented in **Figure 55** would be possible. Potentially 1600 MWh/year electric could be achieved only from the wastewater and biowaste at the facilities of Berger. Combined with the pig manure from the supplying farmers additionally 6000 MWh/year could be achieved. This can reduce the burden on the local electricity grid which becomes especially more important with the increase of renewable energy like sun and wind energy. Additionally, the dependency on natural gas imports can be reduced and increase the stability of the company's energy supply in the future by reducing the risks of changing marked prices. Which has been more frequently the case in the past years induced by the higher likeliness of international conflicts and crisis.

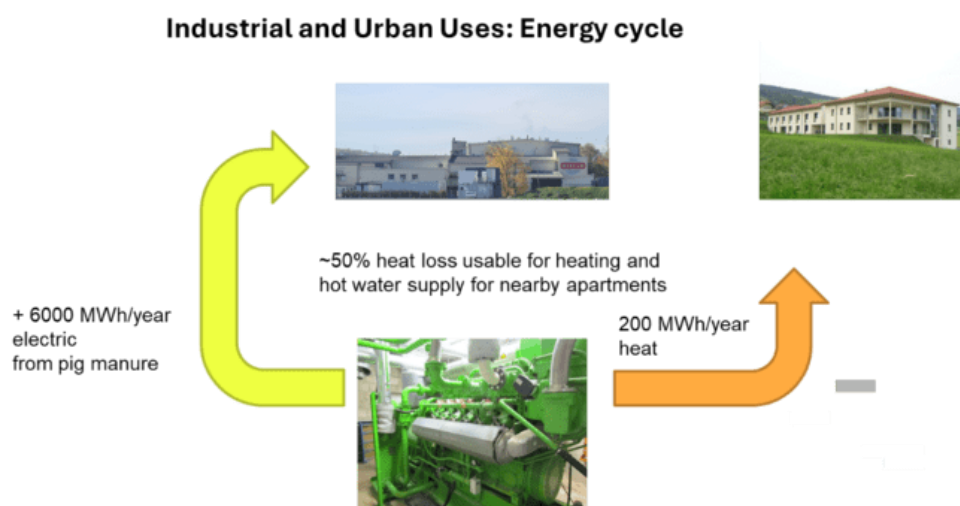


Figure 55. Energy cycle at the Austrian EcoSite

6.2.4 Cleaned water for cooling or irrigation

At the Austrian EcoSite the water treatment system was realized with a flotation unit, because of the high fat content which would have been problematic for membrane systems. The comparative LCA (Described in the WP4 Technical Report) also showed the ecological and monetary benefits compared to an anaerobic membrane system which is more expansive in its realization and more energy demanding in its operation. The flotation water was further treated with an ultrafiltration membrane to gain a particle free solution. Analytical results (Reported in D4.3) showed a very high reduction rate of phosphorous of over 90% removal rates. After ultrafiltration an additional nanofiltration or reverse osmosis step was applied further increasing the water quality almost reaching the highest category of the EU wastewater reuse directive allowing the usage of the recovered water for irrigation of food crops in contact with the water.

Since the Austrian government decided to optout of the EU water reuse directive for now, the focus of the project also shifted towards different water reuse concepts. The most promising is the reuse for cooling water in industrial processes.



The company is producing 180000 m³/year wastewater which has to be treated by the municipal wastewater treatment plant. But the WWTP capacities are almost exceeded because of urban development projects in the region initiated by the installation of a well-connected train station connecting the rural area with the capital of Austria, Vienna. The good connection, lower prices and more nature has led to an increase in population and also wastewater. The treatment and reuse of the company's wastewater would directly effect the WWTP freeing capacities for the increasing demand.

Besides the increasing wastewater amounts in the area, another problem is the decreasing groundwater levels over the past years. A change in precipitation patterns has lead to a higher drought risk and the necessity to irrigate fields with ground water. The area is a highly productive agricultural area producing especially sugar beets. Besides the agricultural use also the municipality has to water trees and recreational areas more frequently. Additionally, there is the practice of private dwells used for the irrigation of gardens and the usage of ground water for pools. All of these increasing demands have lead to a ever decreasing groundwater level. In a recent study the area has been identified to be at a high risk (Greenpeace Austria).

- **Recovered water applications**

The water quality was assessed after Flotation, Ultrafiltration, Nanofiltration and Reverse Osmosis (for details refer to D4.3). The data proved the high reduction rated throughout the process regarding biological and chemical contaminants. The data indicated that all parameters of the EU regulation 2020/741 are met for category A allowing the usage of the water for irrigation of crops intended for human consumption that get in contact with the water (**Table 65**). As Austria has opt out of the regulation the reuse of water for irrigation is difficult and needs permits on a case assessment basis. Potentially the irrigation of 1000-3000 m³/ha/year of sugar beet is possible and in a stakeholder poll 100% of the responses agreed with the irrigation of sugar beets with recovered water, because even though they get in contac with the water the further processing to sugar is very high. This cenario showes overall the highes acceptance rates. The irrigation of the municipal trees, graveyard or the soccer field are also possible applications which would save just for the irrigation of 150 trees 3000 m³/year fresh water.

Table 65. Parameters of the EU regulation for wastewater reuse compared to the values reached after Nanofiltration and Reverse Osmosis.

Parameter	EU REGULATION 2020/741 category A	After Nanofiltration	After Reverse Osmose
E.Coli	<1 log cfu/100 mL	<1 cfu/100mL	<1 cfu/100mL
BOD5	<20 mg/L	48.0 mg/L	4.3 mg/L
TSS	<20 mg/L	1.8 mg/L	0.0 mg/L
Turbidity (NTU)	≤ 5	0.4	0.6

The technically most demanding but most easily implementable, since no permit is necessary, is the reuse as cooling water. The current freshwater demand for cooling in the company is currently 11000 m³/year but expected to increase because of increasing temperatures in the futur. The high risk of no cooling water supply was reported in the past couple of summers with the most alarming beeing



nuclear power plants in France, but also when the cooling systems at food supplying companies is at risk this poses a major danger to our food supply system. The water quality achieved after reverse osmosis would allow the reuse of the water in the cooling system, if the high chloride concentration was reduced by an additional step with an ion exchange or by the reduction of the applied flocculant iron chloride in the flotation process (**Table 66**).

Table 66. Comparison of the parameters required for cooling water and the reached values after reverse osmosis.

Parameter	Requirement cooling water	After Reverse Osmose
pH	6.5 – 9.2	5.8 ± 1.5
Water hardness (CaCO ₃)	50 – 750 mg/L	2.4 mg/L Ca ≡ 5.99 mg/L CaCO ₃
Alkalinity (CaCO ₃)	Max 600 mg/L	22.3 mg/L Ca ≡ 5.99 mg/L CaCO ₃
Dissolved total solids TS	Max 2050 mg/L	0,01 mg/L
Chloride	Max 300 mg/L	1137,8 mg/L
Sulfate	Max 350 mg/L	<1,32 mg S/L
Conductivity	3300 µS/cm	42,5 µS/cm
Suspended solids	Max 25 mg/L	n.a.

Conclusions

The Austrian EcoSite successfully demonstrates an integrated approach to industrial symbiosis. The system enables resource recovery and waste minimization as well as wastewater recovery, supporting circular economy goals.

Table 67. Potential application scenarios in the Austrian EcoSite environment.

Identification of potential applications of treated water/other products/symbiosis at the Austrian EcoSite, Tullnerfeld	Estimated yearly need	Quality required / applicable regulation
Agricultural Uses - Treated water: Irrigation of crops (sugar beet, grapevine, fruit crops, grassland) - Biogas Digestate: fertilizer for farmers/fields - Pig manure: additional future substrate for full scale biogas plant	- Treated water: 1000-3000 m³/ha/year - Biogas digestate: 170-210 kg nitrogen/ha/year - Pig manure: 50 farmers, 64800 pigs/year = 52000 m³/manure/year = around 6000 MWh/year	- Treated water: Austrian Water Rights Act; 1959 (Wasserrechtsgesetz, WRG); EU Regulation 2020/741 on minimum requirements for water reuse - Biogas digestate: national fertilizer regulation (2004 BgBL); EU-regulation 2092/91
Municipal Uses - Treated water: - o Irrigation of young trees - o Irrigation of lawn of soccer field and outdoor pool - Surplus heat of biogas CHP: - o Heating and hot water supply for nearby apartment blocks	- Young trees: around 150 young trees; - Soccer field: around 20-40 L/m²/week; 3000 m³/year - Heating apartments: around 90 apartments; 4000m², 200 MWh	- Treated water: Austria has chosen opt out: EU Regulation 2020/741 on minimum requirements for water reuse
Industrial Uses - Treated water: - o Cooling water for Berger Schinken (BER)	- Cooling water: 40 m³ to 60 m³ cooling water/day; 20000 m³ per year	- Cooling water: EU REGULATION 2020/741 category A



There are several potential application scenarios for the output streams of the Austrian EcoSite summarized in the following **Table 67**. The most promising concepts for now are the usage of the pig manure for biogas production by the farmers supplying the company with pigs and the same farmers as end-user of the produced fertilizer. The recovered water has the highest potential as cooling water if an additional step is implemented for the chloride reduction.

A major hurdle for all concepts is the high density of regulations in the water sector on the one hand, but the missing legislation for biogas production on the other hand making investments in this area a high risk in Austria.



7 GREEK ECOSITE

7.1 Description of the Ecosite

The main objective of the Greek Ecosite is to treat industrial and urban wastewater from the municipality of Western Achaia, along with urban organic solid waste from Ano Alisos. This treatment process aims to produce hydrogen, methane in AnMBR, compost and clean water (see diagram in **Figure 56**).

The advanced wastewater treatment pilot facility consists of multiple interrelated processes:

- Pretreatment of food waste: screw press for hydrogen production and drying shredding for methane production. The biomass generated from the drying/shredding will be led for composting
- Bioreactors: Acidogenic CSTR and methanogenic CSTR in tandem with anaerobic membrane system (AnMBR) for methane production
- Membrane unit: for producing filtered water.

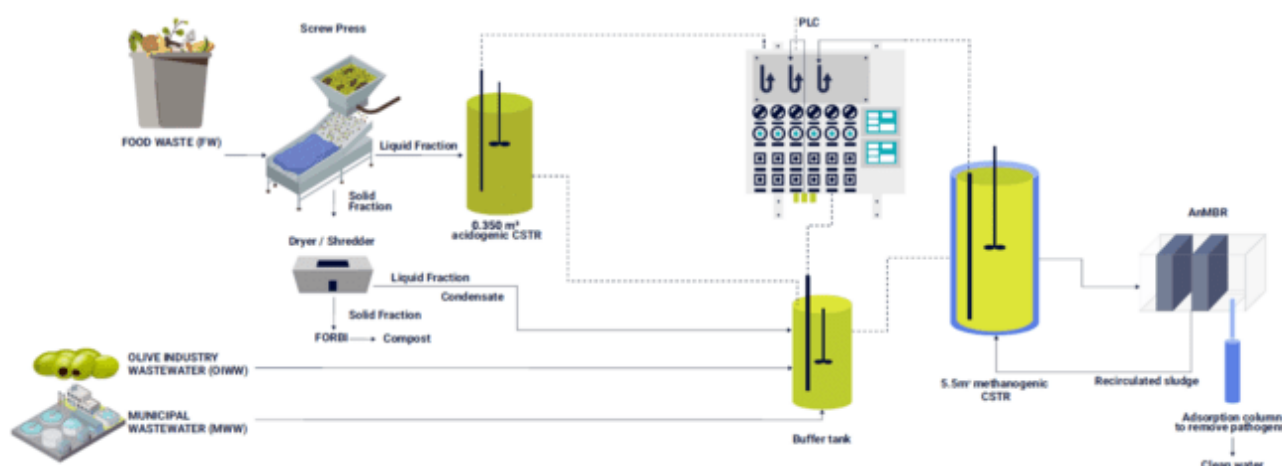


Figure 56. Greek Ecosite: sources of waste, treatment processes and new added-value products

As a result, high-value new resources will be available:

- Biogas rich in methane
- Hydrogen production
- PHAs (lab scale)
- Treated WW for irrigation Compost from the solid phase after pretreatment along with the digestate

7.2 Description of the work (results obtained)

This point describes the obtention and validation of **High Value New Resources (HVNR) of the Greek Ecosite**. This report includes the methodologies applied for resource recovery and validation and examines their technical feasibility, performance, and integration within the urban-industrial symbiosis framework.

The HVNR streams addressed in this deliverable focus on the valorisation of organic and residual feedstocks through complementary biotechnological pathways. Specifically, **hydrogen** and bioplastics (polyhydroxyalkanoates, **PHAs**) are produced via dark fermentation processes, while anaerobic digestion is employed for the production of **biomethane** in a membrane CSTR. Biochar is generated from agro-industrial waste and is utilized in the treatment of anaerobic digestate, in addition to the membrane filtration, enabling the recovery of **water suitable for irrigation** with significantly reduced Critical Emerging Contaminants (CECs), supporting sustainable water management. Finally, **compost** is produced from combinations of olive stone, pruning and Food Organic Residue Biomass (FORBI).

7.2.1 Hydrogen production via dark fermentation

The integration of hydrogen into the energy economy stands as one of the major challenges of the 21st century. Hydrogen offers a promising alternative to fossil fuels, which are approaching their limits, due to its abundance and its potential for clean energy production without harmful environmental emissions. It has a wide range of applications, including the production of industrial chemicals (e.g., NH_3 , HCl , CH_3OH), industrial refrigeration, aerospace technologies, transportation as a fuel, and electricity generation. Among the emerging technologies for sustainable hydrogen production, dark fermentation (DF) is particularly promising, as it combines waste treatment with energy recovery. This process enables the conversion of organic waste into hydrogen, offering a dual environmental and energy benefit.

- **Operation of the dark fermentation system (CSTR1) for hydrogen production**

In the framework of the SYMSITES project, hydrogen production via DF was investigated using household FW collected from the Municipality of Western Achaia. The experiments were conducted in a 350 L pilot-scale reactor installed at the municipal wastewater treatment facility, which is the Greek EcoSite. The process operated under mesophilic conditions (37°C) and within a pH range of 5.5–6. The feedstock consisted of 1) the liquid fraction obtained after the extraction of FW via a screw press (LFFW), and 2) the solid food residues following shredding and drying (FORBI).

Pilot-scale experiments were conducted in the bioreactor at the Greek EcoSite in Western Achaia (Figure 57). The bioreactor had a total volume of 350 L and a working volume of 200 L and was equipped with mechanical agitation and an automatic pH control system, including base dosing. In addition, a heating jacket was installed to maintain a stable operating temperature of 37°C.

The main components of the system included:

- a) Mechanical agitation system
- b) Base dosing system for automatic pH control
- c) Heating jacket
- d) Liquid sampling port





Figure 57. The 350 L pilot-scale reactor (CSTR1) installed at the Greek EcoSite

Analytical methods

In order to control the production process, different analytical methods are applied to measure the following parameters:

- Determination of hydrogen composition
- Determination of volatile fatty acids (VFAs)-Ethanol
- Determination of total and soluble chemical oxygen demand (TCOD and sCOD)
- Determination of carbohydrates
- Determination of solids
- Determination of pH

The D4.4_ANNEX III_NTUA Description of equipment and materials include the description in detail of the different methodologies carried out for the determination of these parameters

• **Hydrogen production process**

Two distinct operational periods were defined: (i) operation using the liquid fraction obtained after the extraction of FW via a screw press (LFFW), and (ii) operation using the solid food residues following shredding and drying (FORBI).

a) Operation using the LFFW

During the first operational period, FW was collected and subsequently processed using a screw press to obtain the liquid fraction, as illustrated in **Figure 58** and **Figure 59**.





Figure 58. Screw process installed in the Greek Ecosite



Figure 59. Extraction process taking place in the screw press of the Greek Ecosite

Small Scale preliminary experiments

Before the operation of CSTR 1, some preliminary experiments were performed to standardize the operational conditions. Thus, two small-scale bioreactors (0.6 L) operated in batch mode, using two different pH controlling approaches. In the first one (batch1), the pH was controlled using a buffer solution of 10.5 g/L NaH_2PO_4 and 14 g/L Na_2HPO_4 , and in the second one (batch2), an online pH controller was provided, simulating the pilot-scale bioreactor CSTR1. The initial conditions of the feeding solution of LFFW are presented in **Table 68**.

Table 68. The main characteristics of the LFFW fed into batch1 and batch2 fermentative bioreactors

pH	6.83
Total carbohydrates (g L ⁻¹)	9.62
Soluble carbohydrates (g L ⁻¹)	8.56
Total COD (g L ⁻¹)	12.64
Soluble COD (g L ⁻¹)	10.78
TSS (g L ⁻¹)	0.93



VSS (g L^{-1}) 0.88

The reactors operated in 2 batches for 60 h each, and the main results regarding Hydrogen, biogas, COD, solids, pH and VFA vs, time obtained are shown in D4.4_ANNEX III_NTUA Description of equipment and materials.

Start-up phase of CSTR1

Thus, the 350 L pilot-scale reactor (CSTR1 located in the Greek Ecosite) operated with online pH control at a constant pH of 5.5. The active volume was 200 L, and the reactor operated in batch mode for almost 70 h. The extract LFFW was used without any chemical addition. Samples of both the liquid and gas phases were collected twice a day, and their composition was analyzed in the lab via the techniques described above. The initial conditions of the feeding solution of LFFW of CSTR1 are presented in **Table 69**.

Table 69. The main characteristics of the LFFW fed into the pilot-scale batch fermentative bioreactor CSTR1

pH	8.51
Total carbohydrates (g L^{-1})	11.21
Soluble carbohydrates (g L^{-1})	10.01
Total COD (g L^{-1})	13.77
Soluble COD (g L^{-1})	12.33
TSS (g L^{-1})	0.97
VSS (g L^{-1})	0.90

The experimental results of the liquid phase analysis are presented below (**Figure 60**. Total and soluble carbohydrate concentrations versus time during the pilot-scale experiment in CSTR1 using LFFW.), where it is clear that both total and soluble carbohydrates were completely consumed.

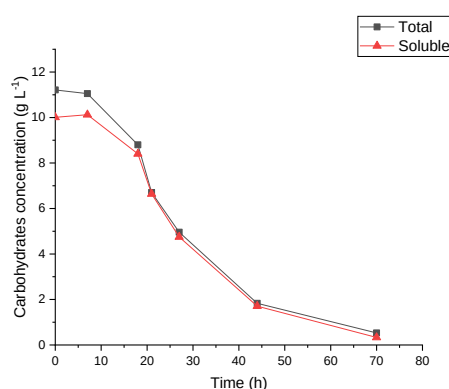


Figure 60. Total and soluble carbohydrate concentrations versus time during the pilot-scale experiment in CSTR1 using LFFW.

The concentrations of COD, as well as TSS and VSS, are presented in Figure 61 and **Figure 62**, respectively. Although the extract obtained from the municipality led to a carbohydrate concentration at the desired value of 10 g L^{-1} , it contained a lower amount of solids compared to the experiments in batch1 and batch2. The COD concentration was very similar to that of the latter.

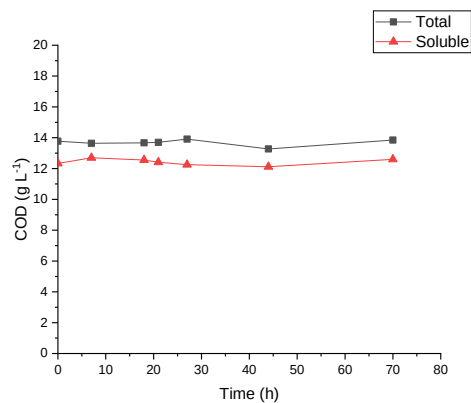


Figure 61. Total and soluble COD concentrations versus time during the pilot-scale experiment in CSTR1 using LFFW.

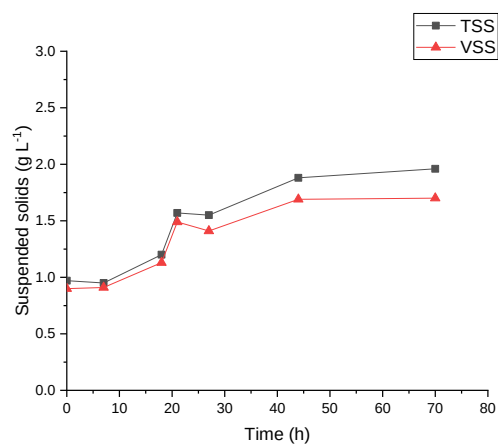


Figure 62. TSS and VSS versus time during the pilot-scale experiment in CSTR1 using LFFW.

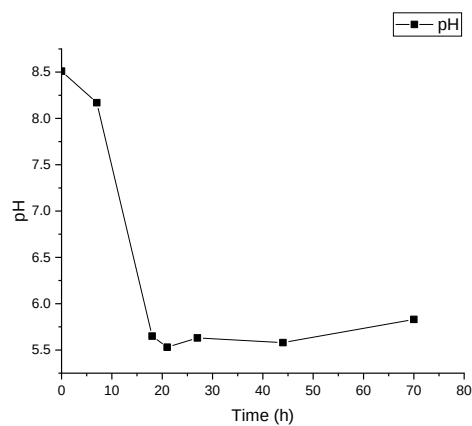
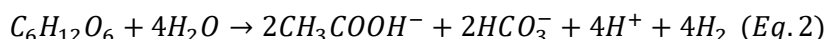


Figure 63. pH versus time during the pilot-scale experiment in CSTR1 using LFFW.

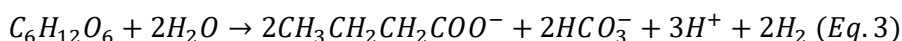
The production of VFAs and ethanol is shown in **Figure 64**. It can be seen that the main metabolic product was butyric acid with a concentration of 2280 mg L⁻¹ in the pilot-scale experiment. Acetic and lactic acids also had high concentrations.

In the gas phase, hydrogen production began shortly after sugar consumption began. The results show that 322 L of hydrogen (H₂) were produced, corresponding to a hydrogen yield of 1.2 mol/mol of sugar consumed. This is a very high hydrogen yield, even surpassing the yield observed in the respective batch hydrogen production experiments. This behavior can be attributed to the presence of microorganisms belonging to the *Clostridium* genus. In this case, the substrate conversion reactions follow the reactions listed below. Based on the theoretical background, the process can yield a maximum of 4 and 2 mol H₂ per mol of glucose when acetic acid and butyric acid are the end products, respectively.



$$\Delta G = -206.3 \text{ kJ mol}^{-1}$$

According to reaction (Eq. 2), the maximum theoretical yield of biohydrogen from glucose fermentation is 4 mol H₂ per mol of glucose consumed. In the case where butyrate is the only organic by-product formed, the maximum theoretical hydrogen yield decreases to 2 mol H₂ per mol of glucose, according to the reaction (Eq. 3).



$$\Delta G = -254.8 \text{ kJ mol}^{-1}$$

Depending on the culture conditions and the type of microorganism, the simultaneous production of both acetate and butyrate is often observed, resulting in intermediate hydrogen yields between 2 and 4 mol H₂ per mol of glucose. Such reactions are characteristic of certain clostridia, such as *Clostridium pasteurianum* (Daesch, & Mortenson, 1968). As also reported in the literature, in practice, hydrogen yields under such conditions typically range between 1–2.5 mol H₂ per mol of glucose, which is consistent with the values obtained in all trials.

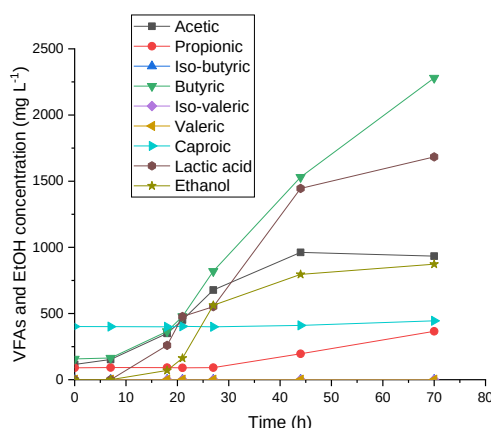


Figure 64. VFAs and ethanol versus time during the pilot-scale experiment in CSTR1 using LFFW.

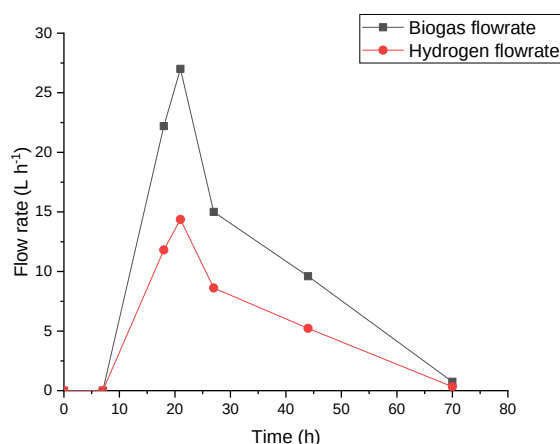


Figure 65. Biogas and hydrogen flow rate versus time during the pilot-scale experiment in CSTR1 using LFFW.

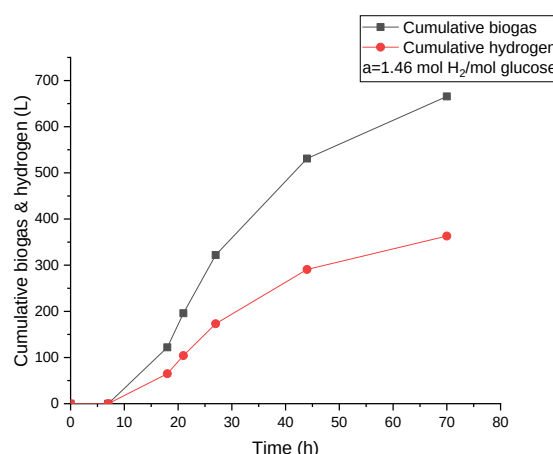


Figure 66. Cumulative biogas and hydrogen production versus time during the pilot-scale experiment in CSTR1 using LFFW.

b) Operating using FORBI

In the Greek EcoSite, DF for hydrogen production was also investigated using FORBI as feedstock. The 350 L pilot-scale reactor (CSTR1, located at the Greek EcoSite) was operated with online pH control, maintaining a constant pH of 5.5. The active volume was 200 L, and the reactor operated in batch mode for nearly 70 h. To prepare the feed, 5 kg of FORBI were suspended in 195 L of water, and 5 L of effluent from the previous pilot-scale experiment were added as the microbial inoculum. The suspension that was produced had the characteristics presented in **Table 70**. Samples of both the liquid and gas phases were collected twice daily, and their composition was analysed in the laboratory, as presented above.

Table 70. Characteristics of the suspension which fed the pilot-scale batch fermentative bioreactor CSTR1

pH	7.66
Total carbohydrates (g L ⁻¹)	4.50
Soluble carbohydrates (g L ⁻¹)	3.58

Total COD (g L ⁻¹)	8.67
Soluble COD (g L ⁻¹)	7.96
TSS (g L ⁻¹)	5.74
VSS (g L ⁻¹)	4.64

The total carbohydrate concentration was within the desired range at 4.5 g L⁻¹. The experiment lasted a total of 70 hours, similar to the other experiments presented above. **Figure 67** up to **Figure 70** schematically present the variation of carbohydrate concentration, COD, TSS, VSS, and pH as a function of time. The variation of carbohydrate concentration, as well as TSS and VSS during the experiment, shows a trend consistent with previous experiments, although in this case the solids content was considerably higher. Of particular interest is the variation of COD, as its continuous increase indicates the hydrolysis of FORBI within the suspension of the feed. **Figure 70**, which illustrates pH as a function of time, confirms the stable operation of the system.

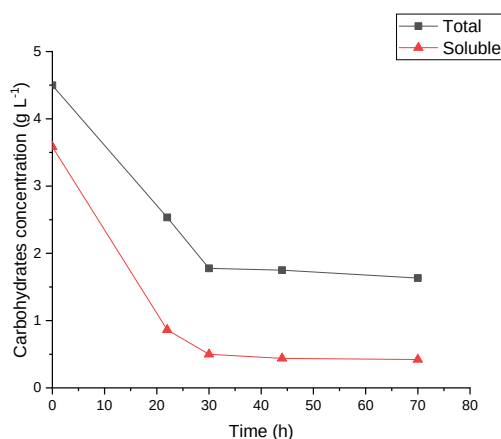


Figure 67. Total and soluble carbohydrate concentrations versus time during the pilot-scale experiment in CSTR1 using the suspension of FORBI.

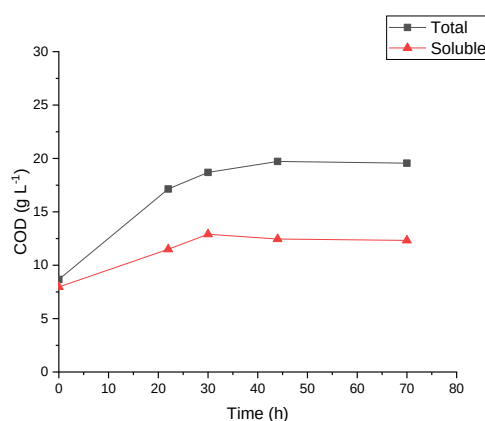


Figure 68. Total and soluble COD versus time during the pilot-scale experiment in CSTR1 using the suspension of FORBI.



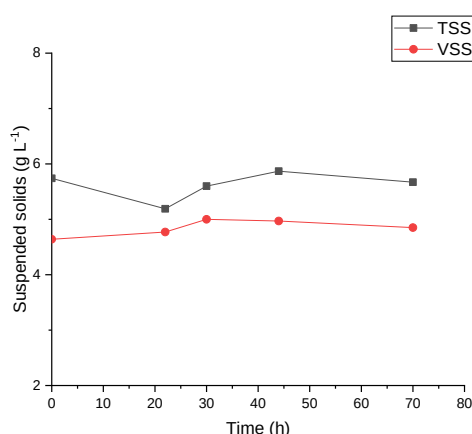


Figure 69. TSS and VSS versus time during the pilot-scale experiment in CSTR1 using the suspension of FORBI.

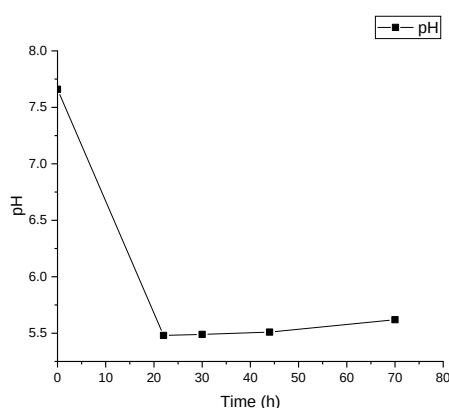


Figure 70. pH versus time during the pilot-scale experiment in CSTR1 using the suspension of FORBI.

Figure 71 illustrates the production of metabolic products as a function of time, revealing notable differences compared to the previous experiments. Although the dominant metabolic pathway remains oriented towards butyric and acetic acid formation, with butyric acid prevailing, there is a clear and significant reduction in the overall production of VFAs as well as ethanol. More specifically, ethanol is practically absent from the mixture, while most VFAs are detected only in negligible concentrations, with the exception of caproic and propionic acid, which are present in small but measurable amounts. This shift in product distribution suggests a modification in microbial activity or substrate utilization under the examined conditions. However, this observation can also be reasonably attributed to the relatively low initial carbohydrate concentration (4.5 g L^{-1}), which limits the overall extent of fermentation and metabolite formation.

Additionally, the reduced accumulation of intermediate products may indicate a more direct conversion pathway or a less active fermentative process overall. These findings highlight the sensitivity of the system to substrate concentration and suggest that operating conditions play a crucial role in determining both metabolic pathways and product yields.

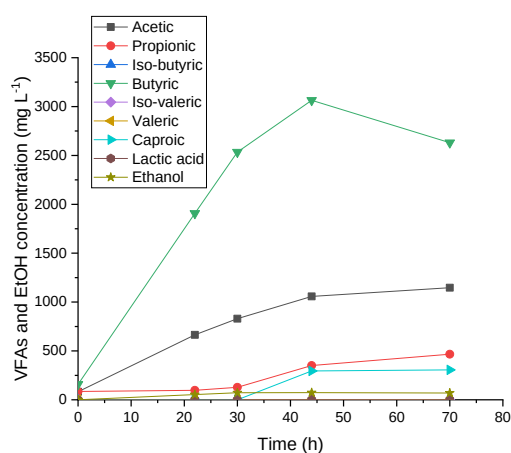


Figure 71. VFAs, lactate, and ethanol versus time during the pilot-scale experiment in CSTR1 using the suspension of FORBI.

The results showed that at the end of the experiments, 320.13 L of H₂ were produced, indicating the high biodegradability of FORBI as a substrate for hydrogen production. In **Figure 72** and **Figure 73**, the biogas and hydrogen production rate and their cumulative productivity are illustrated.

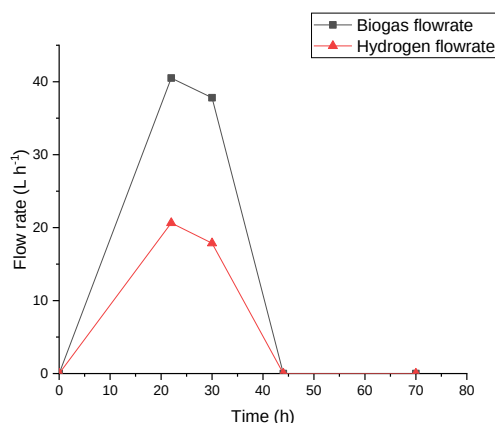


Figure 72. Biogas and hydrogen flow rate versus time during the pilot-scale experiment in CSTR1 using the suspension of FORBI.

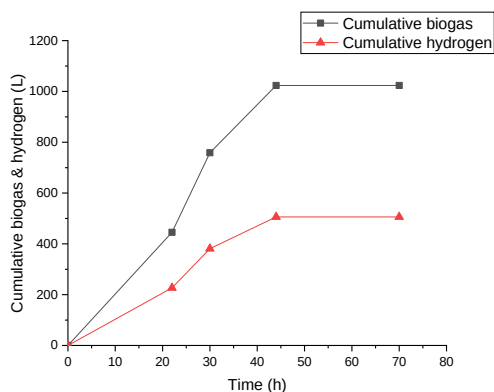


Figure 73. Biogas and hydrogen cumulative production versus time during the pilot-scale experiment in CSTR1 using the suspension of FORBI.



The hydrogen production observed in this experiment is relatively high considering the initial carbohydrate concentration ($\approx 4.5 \text{ g L}^{-1}$). These results can be attributed to three main factors. Firstly, sampling from the upper part of the reactor may have led to elevated measured hydrogen compositions, with values exceeding 50%, due to the highly volatile nature of hydrogen and its tendency to accumulate in the headspace. Secondly, the fact that TSS concentrations did not reach the expected levels suggests a possible inefficiency in the mixing performance of the system, which may have resulted in non-representative liquid sampling. Inadequate mixing could lead to phase separation or uneven distribution of solids, ultimately affecting both analytical measurements and process interpretation. Finally, the potential contribution of alternative metabolic pathways to hydrogen production, beyond carbohydrate fermentation, should also be considered. For instance, hydrogen generation from amino acids could partially explain the high stoichiometric hydrogen yields observed, a phenomenon that has also been reported in the fermentation of industrial FW.

Overall, despite these uncertainties, the system appears promising in terms of producing appreciable amounts of hydrogen, thereby supporting the efficient and comprehensive valorisation of the waste stream.

7.2.2 Exploitation of acidogenic effluents from CSTR1 for the production of PHAs bioplastics

Building on previous work where acidogenic effluents derived from laboratory-scale hydrogen production were successfully utilized as substrates for PHA production. The present study extends this approach to effluents generated at pilot scale. While earlier experiments confirmed the suitability of such streams for bioplastic synthesis under controlled conditions, it is important to evaluate their behaviour when originating from real, large-scale systems, where substrate composition and microbial load are inherently more complex.

In the pilot unit, the effluent from dark fermentation has already been considered for further energy recovery, such as methane production. However, the soluble fraction of this stream, rich in volatile fatty acids (VFAs), also offers the potential for alternative valorization pathways toward higher value-added products, such as bioplastics. In this context, PHA production is not examined as a competing option, but rather as an additional route that could complement existing processes and increase the overall flexibility of the system.

The objective of this work is therefore to assess the suitability of pilot-scale hydrogen fermentation effluents as feedstock for PHA production, and to explore the potential for integrating this process as part of a broader waste valorization strategy. By utilizing the residual carbon present in the effluent, it is possible to enhance resource recovery and move toward a more efficient and holistic use of the initial waste stream. Moreover, this approach contributes to the concept of process coupling, where the output of one biological process serves as the input for another, enabling the development of integrated systems that simultaneously produce energy and materials. Such configurations are particularly relevant in the framework of circular bioeconomy, as they support the transition from single-product to multi-product platforms based on organic waste. An important aspect of this study is the use of real effluents from a pilot-scale system, allowing for a more realistic evaluation of process performance and operational challenges. This is essential for assessing the scalability of the approach and its potential integration into existing infrastructures.

In summary, the experimental work focused on the collection and pre-treatment of pilot-scale effluents from hydrogen production, followed by their use as the sole carbon source in a PHA



production reactor. The system was monitored in terms of substrate consumption, biomass development, and PHA accumulation, while particular emphasis was given to evaluating process stability and performance under real feeding conditions. The results provide initial evidence on the feasibility of converting such effluents into bioplastics and support the potential integration of this pathway within existing waste treatment schemes.

a) Experimental setup

Effluent collection and pre-treatment setup

The effluent from the hydrogen reactor was collected in storage containers and subjected to pre-treatment prior to its use in the PHA reactor. Solid–liquid separation was performed using centrifugation, resulting in a clarified supernatant and a concentrated solid phase (**Figure 74**), aiming to reduce suspended solids and improve substrate homogeneity.

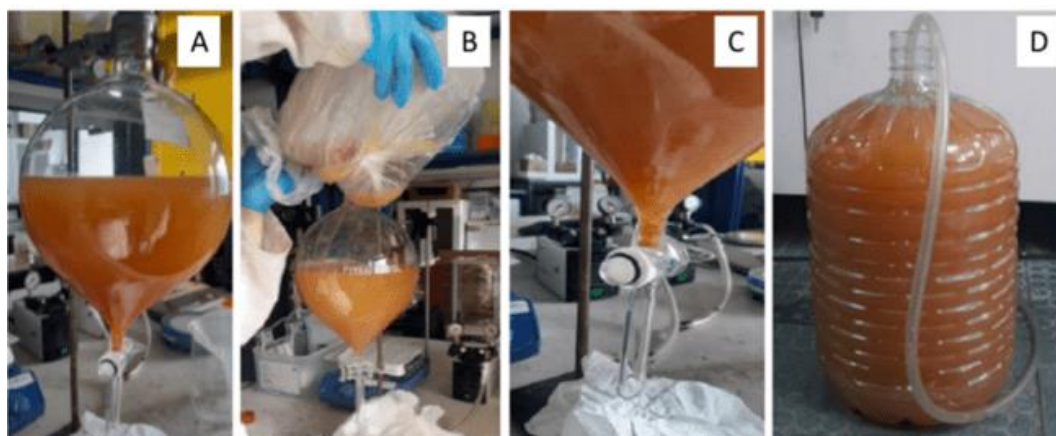


Figure 74. Processing of food waste prior to treatment. (A,B) Phase separation and settling in a separatory funnel; (B) gradual feeding of the raw waste into the funnel; (D) collection of the total processed waste volume (~20 L) for subsequent homogenization.

PHA production reactor (SBR system)

The clarified effluent was used as the sole carbon source in a sequencing batch reactor (SBR) operated under aerobic conditions. The reactor followed a feast–famine regime to promote microbial selection and intracellular PHA accumulation. The system was equipped with aeration, mixing, and controlled feeding, allowing the monitoring of key operational parameters such as pH, biomass concentration, and substrate consumption (**Figure 75**).



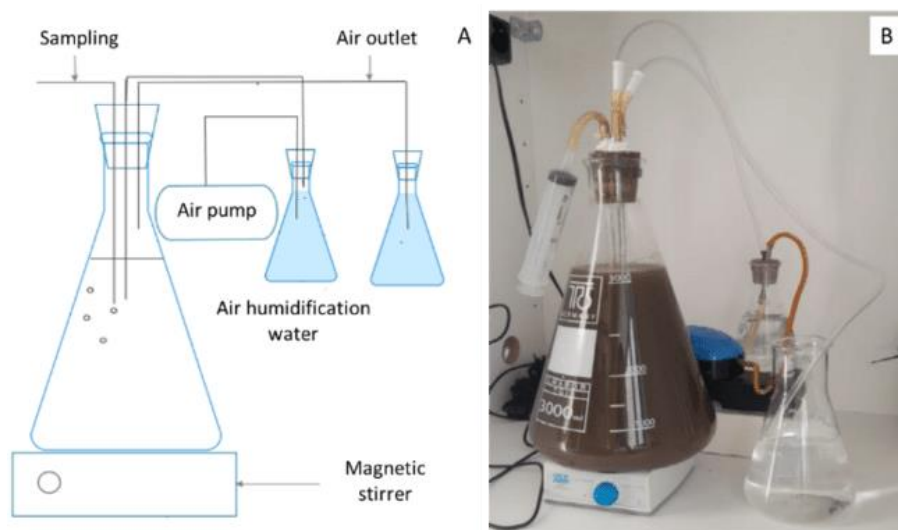


Figure 75. Enrichment reactor of the fill-and-draw type. Schematic representation (A) and actual experimental setup (B).

The system was initially operated using a synthetic feed (**Table 71**) to promote the development of the mixed microbial culture (MMC). This stage ensured controlled conditions for stable microbial enrichment. Following successful MMC establishment, the feed was switched to real wastewater. Specifically, the effluent from CSTR1 was used as the carbon source, as described in **Table 72**. This transition allowed assessment of system performance under more realistic operating conditions.

Table 71. Carbon and nitrogen feed composition (g) for 3 L working volume at $6000 \text{ mg COD L}^{-1}$ and $120 \text{ mg NH}_4^+-\text{N L}^{-1}$.

Carbon source	Amount (g per 3 L)	Nitrogen source	Amount (g per 3 L)
Sodium propionate	2.564		
Sodium butyrate	2.156		
Sodium acetate	1.747	Ammonium chloride	1.375
Sodium lactate	5.242		
Glucose	2.621		

Table 72. Carbon (mL) and nitrogen (g) feed composition for 3 L working volume at $6000 \text{ mg COD L}^{-1}$ and $120 \text{ mg NH}_4^+-\text{N L}^{-1}$ using acidified food waste.

Carbon source	Amount (ml per 3 L)	Nitrogen source	Amount (g per 3 L)
Clarified CSTR1effluent	488.48	Ammonium chloride	1.375

Microbial analysis

For microbial community analysis, biomass samples were collected and processed for DNA extraction and sequencing (NGS). This allowed the identification of microbial populations and the evaluation of their dynamics during reactor operation.

b) Analytical methods

Analytical methods used for the determination of total and soluble Chemical Oxygen Demand (tCOD, sCOD), Volatile Fatty Acids (VFAs), carbohydrates, Total Suspended and Volatile Solids (TSS, VSS) and pH have been described above in section D4.4_ANNEX III_NTUA Description of equipment and materials.

Determination of polyhydroxyalkanoates (PHAs)

The determination of PHAs was carried out using gas chromatography (GC) after acidic digestion of the biomass samples. Prior to analysis, the samples were centrifuged and the collected biomass was dried at 60 °C until constant weight. A known amount of dried biomass (typically 10–20 mg) was subjected to methanolysis in the presence of acidified methanol and chloroform. Specifically, 2 mL of chloroform and 2 mL of methanol containing 3% v/v H₂SO₄ were added to the sample, along with an internal standard solution. The mixture was incubated at 100 °C for 3–4 hours to convert the polymer into its corresponding methyl esters. After cooling to room temperature, 1 mL of distilled water was added to promote phase separation, and the organic phase containing the methyl esters was collected for analysis. The analysis was performed using a gas chromatograph equipped with a flame ionization detector (FID) and a suitable capillary column (e.g., HP-5 or equivalent). A volume of 1 µL of the organic phase was injected into the GC system using an autosampler. The injector and detector temperatures were set at 250 °C and 280 °C, respectively. The oven temperature program started at 60 °C, held for 1 minute, followed by an increase at a rate of 10 °C min⁻¹ up to 220 °C, where it was maintained for 5 minutes. The identification and quantification of PHAs were based on the detection of the corresponding methyl esters (e.g., methyl-3-hydroxybutyrate and methyl-3-hydroxyvalerate) using calibration curves constructed with standard compounds.

Microbial community analysis

The analysis of the microbial community structure was performed through total genomic DNA extraction followed by Next-Generation Sequencing (NGS) targeting the 16S rRNA gene. Biomass samples were collected from the reactor at selected time points and centrifuged to obtain a concentrated pellet, which was stored at -20 °C until further processing. Genomic DNA was extracted using a commercial DNA extraction kit according to the manufacturer's instructions, ensuring efficient cell lysis and purification of high-quality DNA. The extracted DNA was quantified and assessed for purity prior to amplification. The V1–V3 hypervariable regions of the bacterial 16S rRNA gene were amplified using polymerase chain reaction (PCR) with universal primers. The PCR products were then purified and prepared for sequencing according to standard library preparation protocols. Sequencing was performed using a high-throughput platform (e.g., Illumina MiSeq), generating paired-end reads. The obtained sequences were processed using bioinformatics pipelines, including quality filtering, trimming, and clustering into operational taxonomic units (OTUs) or amplicon sequence variants (ASVs). Taxonomic classification was performed using reference databases (e.g., SILVA or Greengenes), allowing the identification of microbial populations at different taxonomic levels. The relative abundance of microbial taxa was calculated and used to evaluate changes in community structure during reactor operation.

c) PHAs production with CSTR1 effluents

Pretreatment of the effluents

Prior to supplying the CSTR1 effluent as carbon source into the PHA production reactor, a pre-treatment step was applied in order to reduce suspended solids and improve the overall stability of the system. The raw effluent, originating from the acidogenic treatment of food waste, contained a significant fraction of particulate matter, which could negatively affect reactor performance by



causing operational issues such as biomass washout, poor mass transfer, and inconsistent substrate availability.

To address this, centrifugation was applied as a simple and effective solid–liquid separation method. The process resulted in a clear reduction of suspended solids, producing a more homogeneous liquid phase suitable for controlled reactor operation. Visual observations confirmed the effectiveness of the separation, with a clear distinction between the clarified supernatant and the concentrated solid fraction. This observation was also supported by quantitative measurements. The comparison of total suspended solids (TSS) and volatile suspended solids (VSS) before and after treatment showed an approximate 30% reduction in suspended solids content, indicating that a significant portion of particulate material was successfully removed (**Table 73**).

Table 73. Characterization of raw and settled food waste.

Parameter	Raw waste (g L ⁻¹)	Settled waste (g L ⁻¹)
COD	12.6	12.3
Carbohydrates	0.53	0.33
NH ₄ ⁺ –N	~0	~0
TSS	1.18	0.85
VSS	1.04	0.74

This reduction is particularly important, as it increases the relative contribution of the soluble fraction of the effluent, which is rich in volatile fatty acids (VFAs) and constitutes the main carbon source for PHA production. From a process perspective, the application of centrifugation offers several advantages. First, it enhances process stability by reducing fluctuations associated with particulate feeding. Second, it improves substrate bioavailability, allowing microorganisms to directly access readily biodegradable compounds. Third, it minimizes operational risks such as clogging, excessive biomass accumulation, and reactor heterogeneity. Although centrifugation is not always the most cost-effective option at large scale, its use in this study demonstrates the benefits of solid–liquid separation. For full-scale applications, simpler alternatives such as gravity settling or primary clarification can achieve comparable solid removal efficiencies with significantly lower energy demand.

Overall, the pre-treatment step contributes significantly to process robustness and reproducibility. The demonstrated reduction of suspended solids, combined with improved substrate quality, supports the feasibility of scaling up the process and integrating it into existing waste treatment infrastructures.

Development of biocatalysts

Prior to the application of real effluent, the microbial culture was developed and stabilized using a synthetic substrate designed to simulate typical acidified waste streams. The aim of this stage was to enrich a microbial community capable of efficient carbon uptake and intracellular PHA storage under controlled conditions. The reactor was operated under feast–famine conditions, promoting the selection of microorganisms with high PHA accumulation capacity. The key results of this stage are



summarized in **Table 74**, highlighting the system's ability to achieve reliable carbon removal and PHA accumulation prior to operation with real waste streams.

Table 74. Key results obtained during development of biocatalysts.

Parameter	Observation / Value	Interpretation
COD removal	~100% (within 72 h)	Complete carbon consumption
NH ₄ ⁺ -N removal	~100% (within 72 h)	Effective nitrogen uptake
Adaptation time	~3 cycles	Biomass acclimation to real wastewater
VFA consumption	Near complete (final cycles)	Efficient substrate utilization
pH range	7.5 – 9.0	Stable and favorable for microbial activity
Biomass (TSS/VSS)	~1.13 (stable)	Good biomass quality and stability
PHA accumulation yield (Y _{PHAs})	~20% (C-based)	Moderate storage capacity
Residual intracellular PHA	~3% after 72 h	Consumption during nitrogen phase
PHA composition	PHBHV (HB:HV ≈ 96:4)	Low HV fraction
System stability	High (after adaptation)	Robust performance under real conditions

Overall, the enrichment strategy successfully established a robust and functionally stable microbial culture, suitable for subsequent transition to real effluent feeding. The system exhibited stable and robust performance after an adaptation period of approximately three cycles under real wastewater feeding. Complete consumption of both carbon and nitrogen sources within 72 h confirmed effective microbial activity and suitable C/N conditions. Efficient VFA uptake and stable biomass characteristics indicated successful acclimation of the MMC. PHA accumulation reached ~20%, demonstrating functional biocatalyst performance, albeit lower than that observed with synthetic feed.

Performance of the PHAs production reactor

Following the pre-treatment of the effluent, the clarified liquid fraction was used as the sole carbon source for the operation of the PHA production reactor. This phase aimed to evaluate reactor performance under realistic feeding conditions, focusing on substrate utilization, biomass dynamics, and PHA accumulation. The reactor was operated under an uncoupled carbon and nitrogen feeding regime, which has been demonstrated to enhance intracellular carbon storage as PHAs in laboratory-scale systems. The system performance and substrate dynamics during operation with acidified food waste are illustrated in **Figure 76** and **Figure 77**, presenting the temporal evolution of COD, NH₄⁺-N, pH, biomass (TSS/VSS), and individual carbon fractions (sugars and VFAs).

As shown, during the operation period, a distinct cyclic behaviour was observed in both COD and NH₄⁺-N profiles (**Figure 76**). COD concentrations reached peaks of approximately 5500–7200 mg L⁻¹ immediately after carbon feeding and were rapidly reduced to near-zero levels within each cycle,



confirming efficient carbon uptake. Similarly, NH_4^+-N concentrations (up to $\sim 130 \text{ mg L}^{-1}$) were fully depleted within short time periods, indicating effective nitrogen assimilation. After the first 2–3 cycles, the system exhibited increased reproducibility in both COD and nitrogen removal, suggesting successful acclimation of the mixed microbial culture (MMC) to the acidified food waste. The pH remained within a relatively narrow and favourable range (7.5–9.2), with no extreme fluctuations observed. Biomass concentrations (TSS and VSS) showed an increasing trend over time, rising from initial values of $\sim 0.8\text{--}1.0 \text{ g L}^{-1}$ to approximately $2.5\text{--}3.0 \text{ g L}^{-1}$, indicating biomass growth and system stabilization. The relatively stable TSS/VSS ratio further supports the maintenance of good biomass quality throughout the operation.

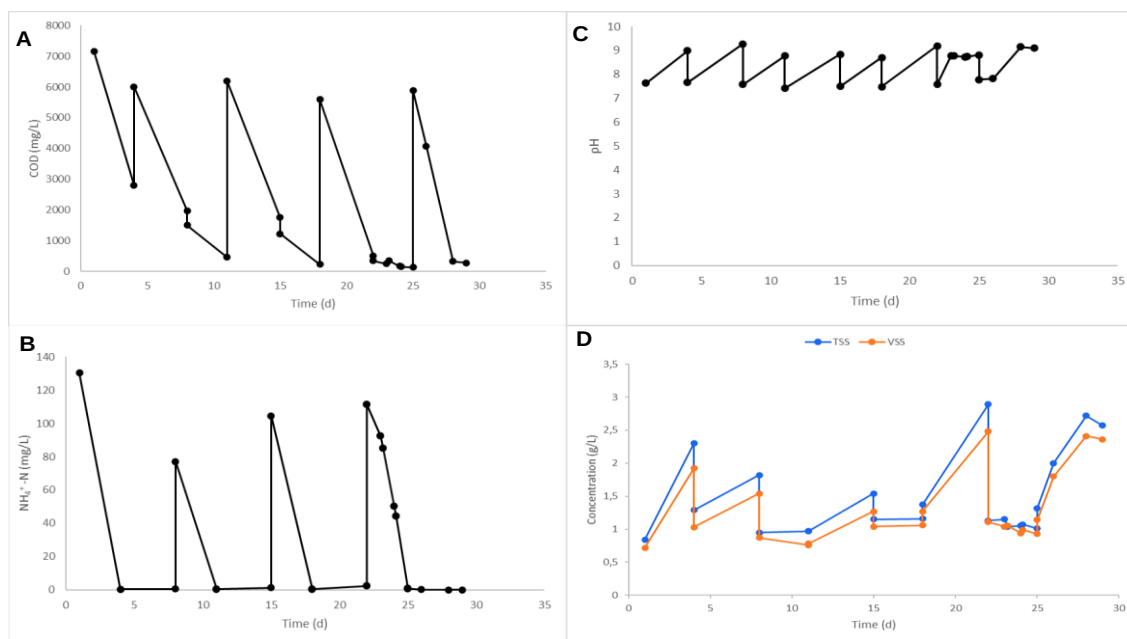


Figure 76. Performance of SBR during operation with acidified food waste (CSTR1settled effluent) as carbon source. (A) COD concentration (mg L^{-1}) over time; (B) NH_4^+-N concentration (mg L^{-1}) over time; (C) pH variation during reactor operation; (D) Biomass concentration expressed as TSS and VSS (g L^{-1}) over time.

The analysis of individual carbon fractions (**Figure 77**) highlights the dynamic consumption of VFAs within each cycle. Acetic acid reached concentrations up to $\sim 700 \text{ mg L}^{-1}$, while propionic acid peaked at $\sim 600 \text{ mg L}^{-1}$. Butyric acid exhibited higher variability, with concentrations reaching up to $\sim 3500 \text{ mg L}^{-1}$, indicating its significant contribution to the overall COD. Caproic acid was also present, with peak values around $\sim 600\text{--}700 \text{ mg L}^{-1}$. In all cases, a rapid depletion of VFAs was observed within each cycle, with concentrations approaching zero, confirming efficient substrate utilization by the MMC.

A strong correlation between VFA consumption and COD removal is evident, indicating that VFAs constitute the primary carbon source driving microbial metabolism. The synchronized depletion of carbon and nitrogen further confirms the effectiveness of the selected C/N ratio (~ 50), supporting both microbial growth and PHA accumulation. Overall, despite the complexity of the real substrate, the system demonstrated stable, resilient, and reproducible performance, highlighting the successful adaptation of the MMC and its potential for operation under realistic conditions.

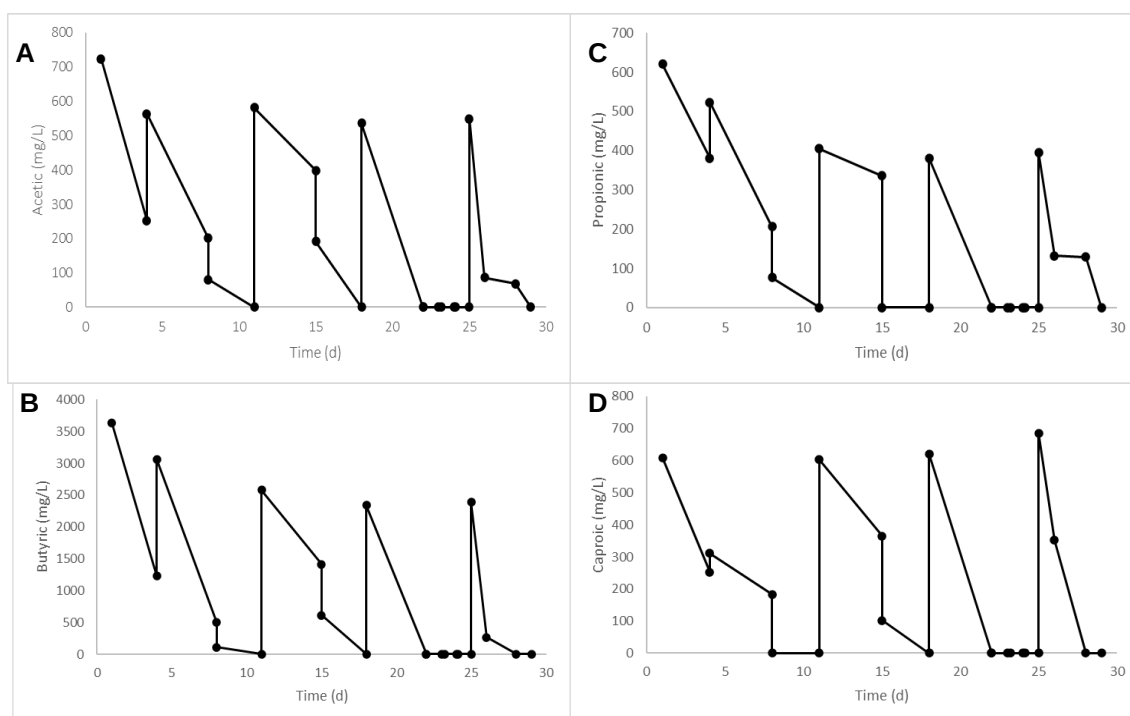


Figure 77. Temporal evolution of soluble carbon compounds during reactor operation with acidified food waste as carbon source. (A) Sugars concentration (mg L^{-1}); (B) Propionic acid concentration (mg L^{-1}); (C) Acetic acid concentration (mg L^{-1}); (D) Butyric acid concentration (mg L^{-1}).

The trends observed in substrate consumption (Figure 76 and Figure 77) are directly reflected in the PHA accumulation behaviour shown in Figure 78. The cyclic availability of carbon and nitrogen governs the intracellular storage and utilization of PHAs, confirming the effectiveness of the uncoupled feeding strategy.

Initially, relatively high PHA contents are observed ($\sim 21 \text{ g PHAs/100 g DCW}$), followed by a decrease during the nitrogen phase, where stored polymers are consumed for cellular maintenance. As the system progresses, a clear and reproducible pattern emerges, with PHA accumulation during the carbon phase (blue lines) reaching values of approximately $14\text{--}18 \text{ g PHAs/100 g DCW}$. During subsequent nitrogen phases (black lines), a gradual decline in PHA content is observed (down to $\sim 10\text{--}12 \text{ g PHAs/100 g DCW}$), indicating polymer utilization. This cyclic behaviour confirms the metabolic switching of the MMC between storage and growth phases.

After approximately the third cycle, the system demonstrates stable and repeatable PHA production patterns, consistent with the previously observed stabilization in COD and VFA consumption. The amplitude between accumulation and consumption phases remains relatively constant, suggesting a balanced metabolic activity and efficient regulation of intracellular carbon storage. The gradual decrease of residual PHA during nitrogen phases, particularly in later cycles, also indicates effective biomass adaptation and improved substrate utilization efficiency.

When compared to the operation with synthetic feed (SBR), a slightly lower PHA accumulation capacity is observed in the present system. While synthetic substrates typically resulted in higher and more consistent storage yields, the use of acidified food waste introduces greater substrate complexity and variability, which likely affects carbon availability and uptake rates. Nevertheless, the system maintains stable and reproducible performance, demonstrating the robustness of the MMC. Importantly, despite the lower maximum yields, the ability to sustain cyclic PHA accumulation under

real wastewater conditions highlights the practical applicability and scalability potential of the process.

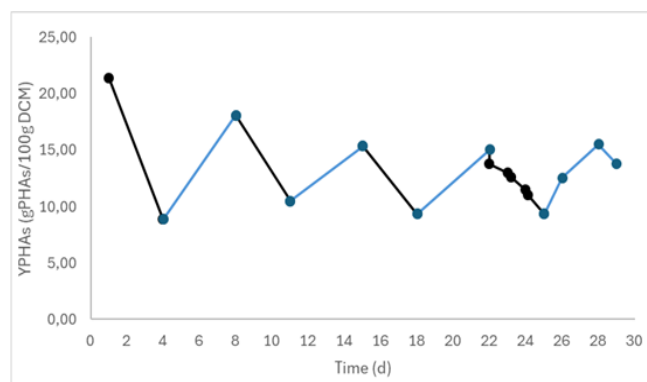


Figure 78. Temporal variation of PHA accumulation (YPHAs, g PHAs/100 g DCW) during reactor operation with acidified food waste. Blue lines indicate the carbon-rich phase (PHA accumulation), while black lines represent the nitrogen phase (PHA consumption for growth and maintenance).

The PHA composition was analysed via GC-FID and was identified as a PHBHV copolymer with an approximate monomeric composition of 96:4 (HB:HV). The relatively low HV fraction can be attributed to the composition of the carbon substrate, as acidified food waste is typically dominated by even-chain VFAs, particularly acetic and butyric acids, which primarily favour PHB formation. In contrast, the incorporation of HV monomers is mainly associated with the presence of odd-chain carbon sources such as propionic acid. Compared to the synthetic feed, where a higher HV fraction (90:10) was observed, the lower HV content in this system suggests a reduced availability or uptake of propionate or other HV precursors. This difference highlights the influence of substrate composition on PHA monomer distribution and indicates that, under real wastewater conditions, the polymer tends to be more PHB-rich, potentially affecting its material properties.

Microbial community analytical results

Figure 79 illustrates the evolution of the microbial community at genus level, highlighting the adaptation of the MMC to wastewater feeding and the impact of SBR operational conditions on the selection of PHA-accumulating microorganisms.

As shown in the first diagram (**Figure 79A**), the microbial community structure highlights the strong influence of both wastewater composition and the settling process on community selection. The initial acidified wastewater (AW.Mix) is dominated by typical fermentative and acidogenic genera such as *Clostridium* (~15%), *Enterococcus* (~21%), *Klebsiella* (~11%) and *Hoylella* (~10%), reflecting the anaerobic, carbohydrate-rich conditions of acidogenic fermentation. Following settling (AW.MixClr), a clear shift is observed, with a marked increase in *Clostridium* (~34.6%) and a general decrease in most other genera. This suggests that settling not only removes solids (~30%) but also selectively retains suspended, spore-forming and strictly anaerobic microorganisms, while bacteria associated with particulate matter (e.g., lactic acid bacteria, *Klebsiella*) are partially removed. Therefore, the process acts as a physicochemical and ecological selection step, enriching the liquid fraction in functionally relevant acidogenic bacteria.



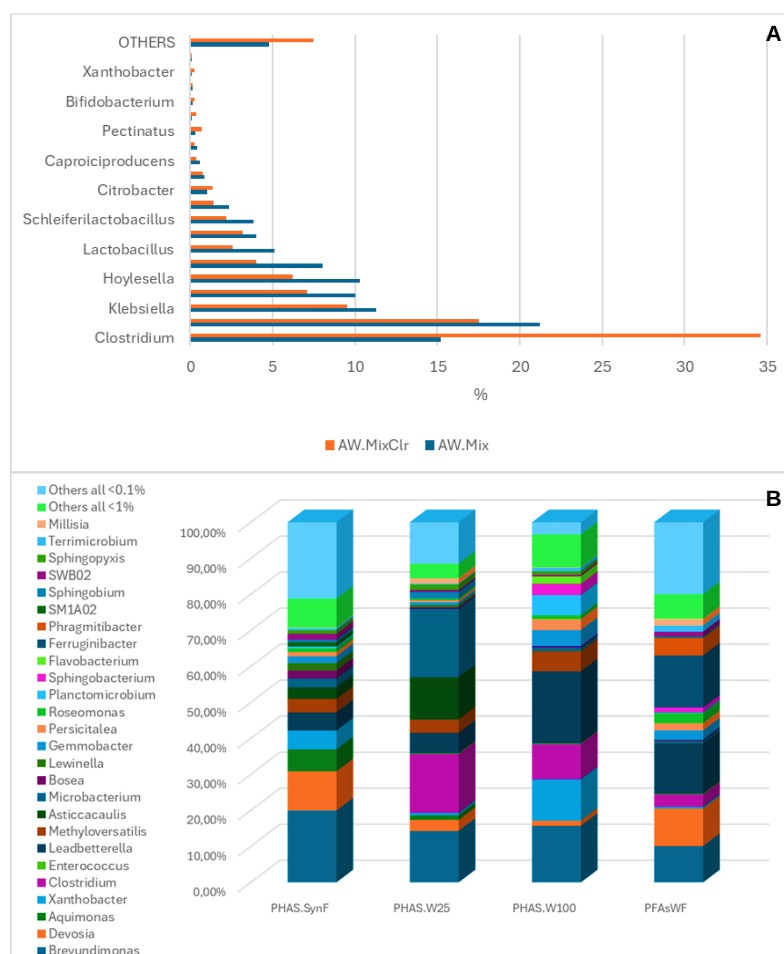


Figure 79. Microbial community composition at genus level based on NGS analysis. (A) Relative abundance (%) of dominant genera in the CSTR1 effluent before (AW.Mix, blue) and after settling (AW.MixClr, orange). (B) Microbial community structure (%) during different operational phases of the SBR: PHAs.SynF (end of operation with synthetic feed), PHAs.W25 (25% substitution with settled CSTR1 effluent), PHAs.W100 (75% substitution), and PFAsWF (final cycle of the SBR operated with 100% settled CSTR1 effluent).

The evolution of the microbial community during SBR operation (**Figure 79B**) further demonstrates the progressive adaptation of the mixed microbial culture (MMC) to the transition from synthetic to real substrate. Under synthetic feeding conditions (PHAs.SynF), the community is dominated by aerobic and facultative genera associated with PHA accumulation, such as *Brevundimonas* (~20%), *Devosia* (~10.9%) and *Aquimonas* (~6.1%), indicating strong selection for fast substrate uptake and storage capacity. Upon partial substitution with wastewater (PHAs.W25), a transitional community emerges, characterized by a sharp increase in *Clostridium* (from ~0.03% to 16.3%) and enrichment of genera such as *Microbacterium* (~19%) and *Asticcacaulis* (~11.8%), while maintaining a significant fraction of the original aerobic population (*Brevundimonas* ~14.3%). This reflects the introduction of fermentative microorganisms from wastewater and the coexistence of aerobic and anaerobic metabolisms. With full substitution (PHAs.W100), the community reorganizes towards genera capable of utilizing more complex substrates, including *Leadbetterella* (~20.1%), *Brevundimonas* (~15.7%), *Xanthobacter* (~11.5%) and *Clostridium* (~9.8%), indicating adaptation to substrate heterogeneity. Finally, after prolonged operation (PFAsWF), a stable and functionally adapted consortium is established, dominated by *Ferruginibacter* (~14.5%), *Leadbetterella* (~14.1%), *Devosia* (~10.4%) and *Brevundimonas* (~10.1%), while *Clostridium* decreases to ~3.4%.

reflecting the dominance of aerobic and microaerophilic organisms under SBR conditions. The transient enrichment of *Microbacterium* in PHAs.W25 followed by its decline further supports the existence of intermediate adaptation stages.

Overall, despite increased community complexity (as indicated by the contribution of low-abundance genera), the system maintains functional stability, demonstrating that the MMC can successfully adapt to real wastewater while preserving its PHA production capability.

7.2.3 Biochar from olive stones

Olive stone waste provided by partner Elaiourgikes Epiheiriseis Patron S.A. (EEP); olive oil industry, were sent to partner GREENE for production of biochar through pyrolysis. The olive stones were pyrolyzed in a pilot plant in the GREENE's facilities, **Figure 80**:



Figure 80. Pilot plant used for pyrolysis treatment: scheme (right) and biomass used for biochar production (olive stones) (left).

The operational parameters were:

- Pressure: atmospheric pressure
- Flow: Inert atmosphere
- Time: 2 h
- Temperature: 550 °C

The produced biochar was sent back to the Greek ECOSITE and its performance on water purification and critical emerging contaminants removal was evaluated. Results are presented in detail in the corresponding section of Deliverable 4.3.

7.2.4 Methane production

Methane was produced from CSTR 2 during the symbiotic treatment of industrial and urban waste streams. The system operated under 6 distinct operational phases featuring feedstock combinations of Municipal WasteWater (MWW), Olive Industry WasteWater (OIWW), Liquid Fraction of Food Waste (LFFW) after screw press and Condensate (liquid stream from dryer/shredder of solid food waste). The high rate AnMBR system was operated successfully in very low HRT of 1 to 4 d maintaining high organic load removal rates. Detailed results regarding system operation and monitoring are presented in Deliverable 4.3.

Observations from those operational periods demonstrate clear trade-offs between methane production, process intensification (HRT) and effluent quality. Period 3 (MWW+OIWW+LFFW, HRT 4 d) achieved moderate methane production (up to 2.6 m³/d) but resulted in poor effluent quality



(COD >1 g/L). In contrast, Period 2 (MWW+OIWW, HRT 2 d) showed the highest methane yield (up to 3.6 m³/d) alongside significantly improved effluent quality (~300 mg/L COD). Period 4 (MWW+OIWW+condensate, HRT 4 d) exhibited lower methane production (up to 2 m³/d) but achieved excellent effluent quality (COD <100 mg/L), fully complying with legislative limits. Finally, Period 6 (MWW+OIWW, HRT 1 d) maintained relatively moderate methane production (up to 2.6 m³/d) despite the reduced HRT, with effluent COD (~130 mg/L) close to regulatory thresholds, demonstrating efficient reactor performance under intensified conditions. Overall, Period 2 appears optimal for energy recovery at the cost of higher effluent COD, while Period 4 provided the best effluent quality and overall performance combining all 3 waste streams, and Period 6 reflects a promising high-rate operation (HRT 1d) with acceptable performance.

7.2.5 Compost

This study investigated the composting performance of three distinct organic feedstocks under controlled aerobic conditions to evaluate decomposition rates, physicochemical changes, and final compost quality. Namely the 3 feedstocks are olive stones, pruning and FORBI (after drying and shredding of food waste - D.4.3). 3 composting experiments were carried out with the following mixture characteristics:

- COMPOST 1: 50% pruning and 50% olive stone
- COMPOST 2: 50% pruning and 50% FORBI
- COMPOST 3: 34% pruning, 33% olive stone, 33% FORBI

• Composting setup

Each mixture was composted in composting bins (250L capacity - **Figure 81**) containing approximately 200L of raw material at start-up (filling rate 80% v/v). All bins were established outdoors under a rain shelter to minimize the influence of precipitation. The experiment was conducted over a 60-65 day period.



Figure 81. Setup of compost 1, 2 and 3 from left to right in the 250L compost bins.

Piles within the bins were turned manually every 2 days for the duration of the experiments to maintain proper aeration within the compost mixture. Moisture content was monitored every 2 days as well and water was added to maintain a moisture content of approximately 50-60% w/w. Temperature was monitored at 5 points of each pile and recorder every 2 days. Total and Volatile solids, pH and conductivity (measured in a 1:10 w/v water extract) were also monitored every 2 days.

- **Compost experiment 1: 50% pruning and 50% olive stone**

Average and maximum temperatures for Compost 1 within the duration of the experiment are presented in **Figure 82**. Both average and maximum temperatures rise rapidly at the onset of the composting process, with T max reaching 60-64°C and T avg around 51-54°C within the first 6-8 days, indicating vigorous microbial thermophilic activity driven by the readily degradable organic fractions in the pruning and olive stone mixture. This thermophilic phase, where T max consistently exceeds 55°C, is sustained through approximately day 8, satisfying the hygienic requirements typically associated with effective composting. A sharp decline in both parameters follows from day 10 onwards, with T avg dropping to 34-39°C and Tmax to 44-49°C by days 13-20 and continuing to fall steadily through the mesophilic range, reaching 22-25°C and 25-27°C respectively around days 30-40 as the organic matter becomes depleted. From day 45 onwards, both T avg and T max converge and decline further toward ambient temperatures of ~15-18°C by days 55-65, indicating the maturation phase of the composting process where microbial activity has largely subsided. The error bars on T avg are notably wider during the thermophilic and early cooling phases, reflecting greater spatial or temporal temperature variability within the pile during active decomposition, which narrows considerably in the later stages as the pile equilibrates thermally with the environment.

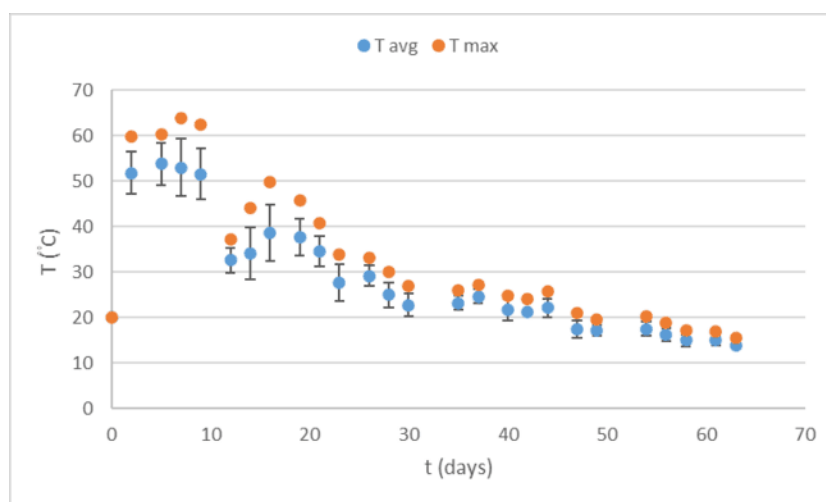


Figure 82. Average and maximum temperatures (°C) recorded in Compost Pile 1 (50% pruning, 50% olive stone) over the composting period

Total and Volatile Solids (TS, VS) for Compost 1 within the duration of the experiment are presented in **Figure 83**. Total solids (TS) begin at 50% w/w on day 1 and rise to a peak of ~60% by day 12, likely reflecting moisture evaporation driven by the intense thermophilic heating observed during the same early phase, despite the frequent addition of water. A notable drop follows around days 15-22, with TS falling to 40-46% within the pile as temperatures declined. TS then fluctuates moderately between 46-51% through days 25-37 before stabilizing in the 42-48% range from day 47 onwards, suggesting the pile approached a more consistent moisture equilibrium during the maturation phase. Volatile solids (VS) closely track TS throughout the entire experiment, starting at ~47% and gradually

declining in the early phase to 38-41% by the end of the run, reflecting the progressive mineralization and decomposition of organic matter over time.

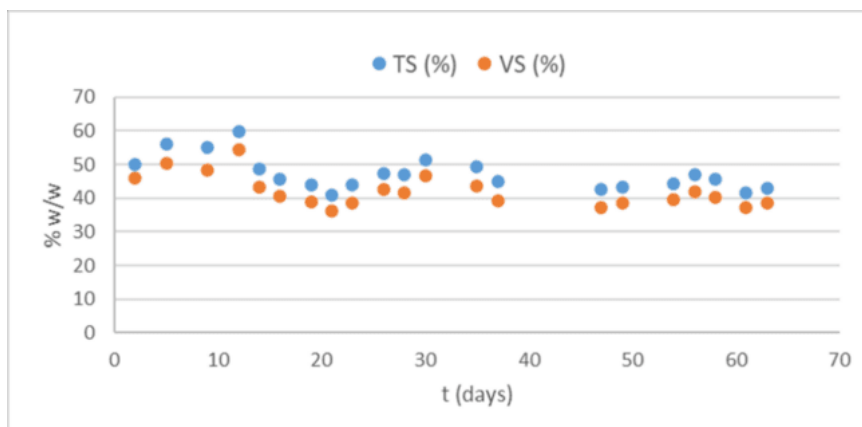


Figure 83. Total solids (TS) and volatile solids (VS) (% w/w) in Compost Pile 1 (50% pruning, 50% olive stone) over the composting period.

Conductivity and pH for Compost 1 within the duration of the experiment are presented in **Figure 84**. Compost pH starts at ~7.3 on day 1 and rises rapidly to ~8.2-9.2 by days 6-9, reflecting the early alkalization commonly associated with the thermophilic phase of composting, where ammonia volatilization from protein and nitrogen-rich organic matter drives pH upward. A further increase brings pH to ~9.5-10.0 between days 13-23. From day 25 onwards, pH stabilizes remarkably consistently in the ~9.4-9.8 range through day 63, showing very little variation despite the significant changes in temperature, moisture, and volatile solids occurring concurrently. The sustained alkaline pH throughout the maturation phase is characteristic of olive-derived composting substrates, where the buffering capacity of the mineral-rich olive stone fraction likely contributes to maintaining elevated pH values. The electrical conductivity (EC) of the compost remained relatively stable throughout the 63-day composting period, fluctuating within a narrow range of approximately 1.15 to 1.75 mS/cm. EC values started at around 1.5 mS/cm and showed a slight increase during the early thermophilic phase (Days 2-9), reaching a peak of ~1.75 mS/cm, reflecting the rapid mineralization of organic matter and release of soluble ions. Following this peak, a gradual declining trend was observed through the remainder of the process, with values stabilizing between 1.2-1.4 mS/cm in the maturation phase (Days 45-63).

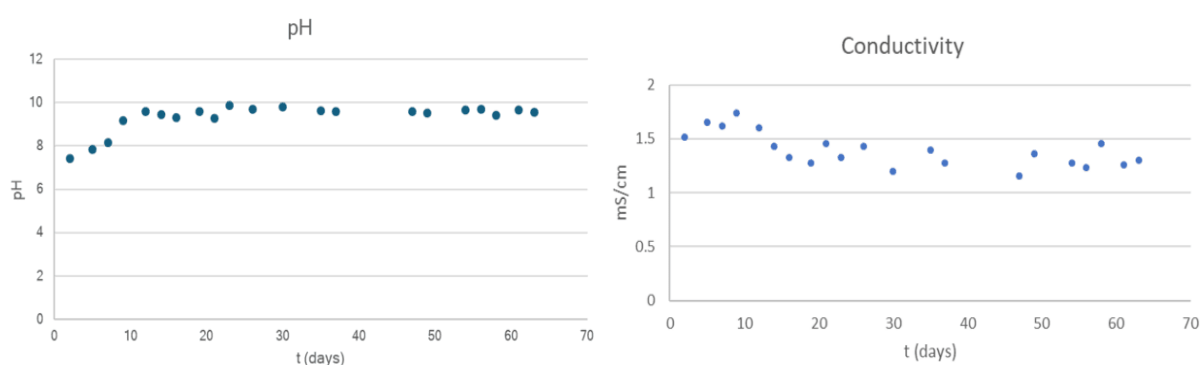


Figure 84. pH evolution and conductivity of Compost Pile 1 over the composting period.

- **Compost experiment 2: 50% pruning and 50% FORBI**

Average and maximum temperatures for Compost 2 within the duration of the experiment are presented in **Figure 85**. Both average and maximum temperatures begin at notably high values from day 1, with T avg at 54°C and T max at 58°C, suggesting that the thermophilic phase was already well underway at the first measurement point, likely initiated within the first day of composting. T max remains elevated at 59-61°C through days 7-10 while T avg fluctuates around 52-53°C during the same period, sustaining thermophilic conditions above 55°C for an extended duration and ensuring effective pathogen reduction. A decline begins around day 16, with T avg dropping to 33°C and T max to 49°C, marked by notably wide error bars suggesting considerable spatial temperature heterogeneity within the pile at this transition point. Both parameters then continue declining steadily through the mesophilic range, with T avg reaching 22-29°C and T max 29-35°C between days 22-32, before converging further toward 18-22°C by days 45-50. From day 55 onwards, both T avg and T max stabilize near ambient temperatures of ~15-20°C through day 65, indicating entry into the final maturation phase.

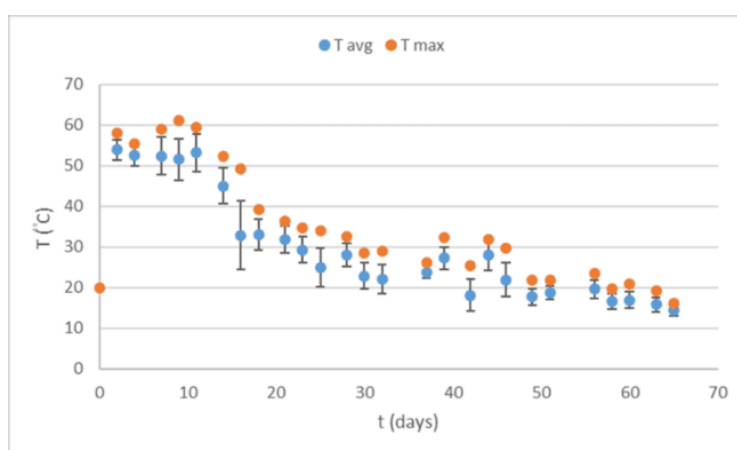


Figure 85. Average and maximum temperatures (°C) recorded in Compost Pile 2 (50% pruning, 50% FORBI) over the composting period

Total and Volatile Solids (TS, VS) for Compost 2 within the duration of the experiment are presented in **Figure 86**. Total solids (TS) begin at 51% w/w on day 1 and rise to a peak of 67-69% by days 8-15, again reflecting significant moisture loss driven by the intense thermophilic heating documented during the same early phase. A sharp drop follows around day 19-20, with TS falling to 38-40%. TS then fluctuates moderately between 45-57% through days 22-37 before declining more consistently to ~40% from day 55 onwards, suggesting progressive stabilization of the pile's moisture content during maturation. Volatile solids (VS) closely track TS throughout, starting at 48% and peaking at 60% in the early thermophilic phase before declining gradually to 30-32% by the end of the run, reflecting sustained organic matter mineralization over the composting period.

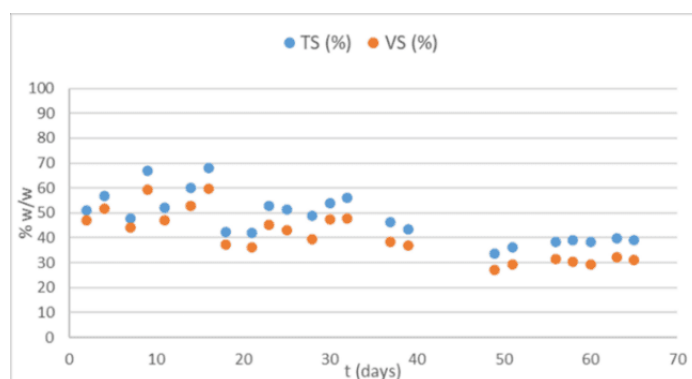


Figure 86. Total solids (TS) and volatile solids (VS) (% w/w) in Compost Pile 2 (50% pruning, 50% FORBI) over the composting period

Conductivity and pH for Compost 2 within the duration of the experiment are presented in **Figure 87**. Compost pH starts at ~6.0 on day 1 and remains relatively low and stable around 5.8-6.3 through day 12, suggesting an initial slightly acidic environment possibly driven by the early release of organic acids during the onset of microbial decomposition of FORBI. A steady rise follows from day 13 onwards, with pH climbing to 7.7 by day 15 and continuing to increase to 8.2-8.5 by days 20-25, reflecting the shift toward alkaline conditions as ammonia volatilization and organic acid consumption by thermophilic microorganisms progress. From day 25 onwards, pH stabilizes remarkably consistently in the 8.0-8.5 range through day 65, indicating a well-buffered and chemically stable system during the maturation phase. The electrical conductivity (EC) of this compost remained stable throughout the entire 65-day composting period, consistently ranging within a narrow band of approximately 2.0 to 2.5 mS/cm. Values started at 2.3 mS/cm and showed only minor fluctuations during the active thermophilic phase (Days 2-10), with a slight peak of ~2.4 mS/cm around Day 8, likely associated with the initial release of soluble ions from mineralizing organic matter. Throughout the maturation phase (Days 40-65), EC stabilized consistently around 2.1–2.2 mS/cm, indicating ionic equilibrium was reached.)

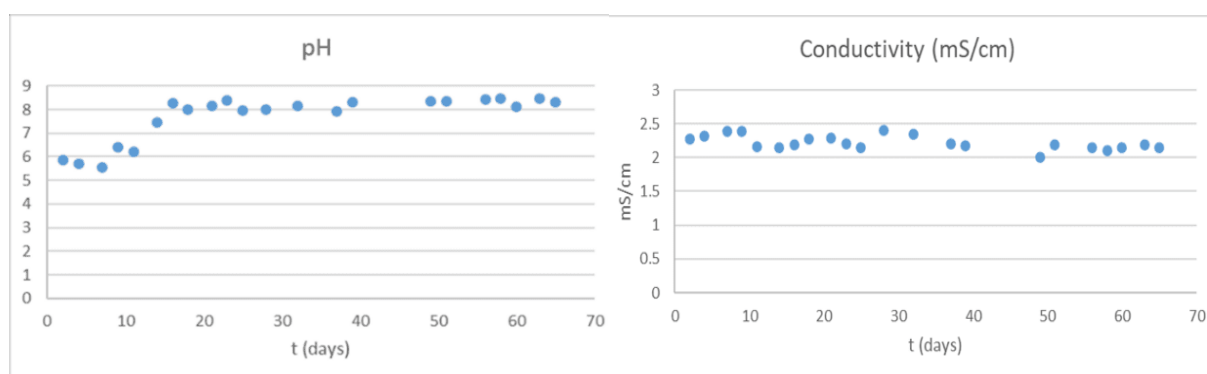


Figure 87. pH evolution and conductivity in Compost Pile 2 over the composting period).

- **Compost experiment 3: 34% pruning, 33% olive stone, 33% FORBI**

Average and maximum temperatures for Compost 3 within the duration of the experiment are presented in **Figure 88**. Both average and maximum temperatures rise quickly from the start, with T avg reaching ~60°C and T max 63°C in the first 3 days, establishing a prolonged and robust thermophilic phase that is sustained for nearly three weeks - notably longer than in the previous two compost piles. T max briefly peaks at ~65°C around day 18, the highest value recorded across all

three composting mixtures, before a sharp decline begins around days 19-20, where T_{avg} drops steeply to 45-50°C and T_{max} to 57-60°C, wide error bars at that point reflecting high spatial temperature variability within the pile. Both parameters then decline steadily through the mesophilic range, with T_{avg} falling to 32-35°C and T_{max} to 43-48°C between days 25-35, continuing downward to 25-30°C by days 37-42. From day 47 onwards, both T_{avg} and T_{max} converge and stabilize near ambient temperatures of 18-22°C through day 61, signalling the onset of the final maturation phase. The extended duration of the thermophilic phase and the higher peak T_{max} compared to the other two piles suggest more vigorous initial microbial activity in this mixture, potentially reflecting a more favourable substrate composition thanks to the combination of all 3 feedstocks.

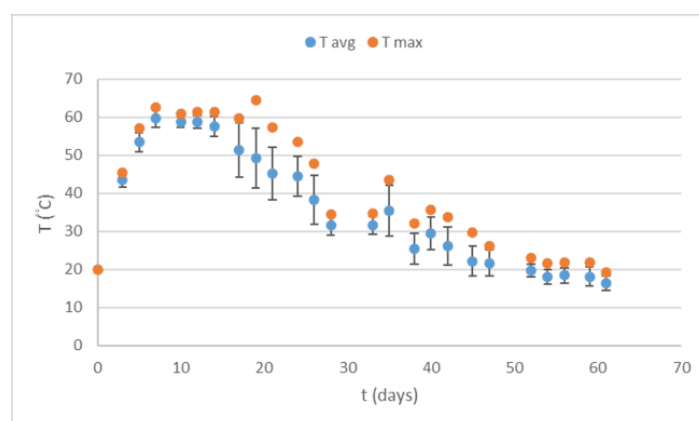


Figure 88. Average and maximum temperatures (°C) recorded in Compost Pile 3 (34% pruning, 33% olive stone, 33% FORBI) over the composting period

Total and Volatile Solids (TS, VS) for Compost 3 within the duration of the experiment are presented in **Figure 89**. Total solids (TS) begin at 50% w/w on day 1 and rise modestly to 57-59% by days 8-10, reflecting early moisture evaporation during the thermophilic phase, though the peak is less pronounced than in the previous two compost piles despite this mixture exhibiting the longest and most intense thermophilic phase. TS then fluctuates in the 45-57% range through days 13-35, with a notable dip to 40-47% around days 24-27 before a brief recovery to ~57% around day 35. From day 45 onwards, TS declines more consistently to 40-50%, showing moderate variability through day 61. Volatile solids (VS) track TS closely throughout, starting at ~49% and remaining in the 43-52% range during the active phase before declining to 38-42% from day 45 onwards, indicating progressive but moderate organic matter mineralization during maturation. Compared to the other two compost piles, this mixture displays a narrower overall range in both TS and VS, with less dramatic peaks and a more gradual declining trend, and the final VS values of 38-42% are intermediate between the first pile (38-42%) and the second pile (30-35%), suggesting a moderate degree of organic matter degradation relative to the other mixtures despite its more vigorous thermophilic phase.



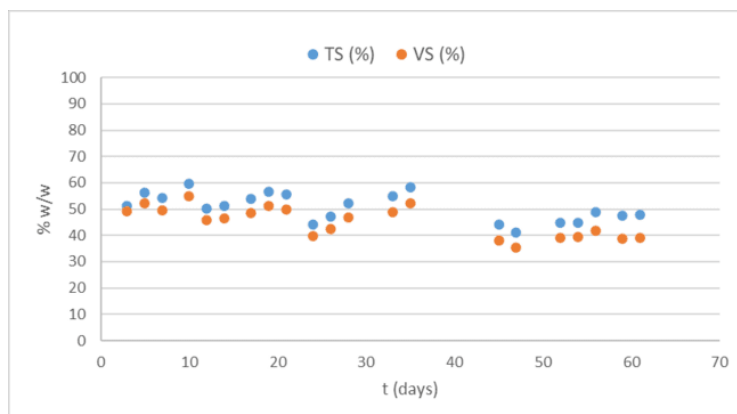


Figure 89. Total solids (TS) and volatile solids (VS) (% w/w) in Compost Pile 3 (34% pruning, 33% olive stone, 33% FORBI) over the composting period.

Conductivity and pH for Compost 3 within the duration of the experiment are presented in Compost pH starts at ~5.0 on day 1, the lowest initial value recorded across all three compost piles, and rises gradually through 5.3-6.5 between days 5-13, reflecting a more pronounced initial acidic phase likely driven by the accumulation of volatile fatty acids during early fermentation of the substrate. A steady and continuous increase follows from day 15 onwards, with pH climbing through 6.7-7.5 by days 18-22 and continuing to rise to 8.7-9.0 by days 25-30, indicating progressive ammonia-driven alkalization as thermophilic decomposition intensifies. pH then stabilizes at 9.0-9.5 from day 32 onwards through day 61, showing very little variation during the maturation phase. The electrical conductivity (EC) of this compost was highly stable throughout the 61-day composting period, fluctuating within a narrow range of approximately 2.0 to 2.4 mS/cm. EC values started at 2.2 mS/cm and remained relatively flat during the early stages, with a modest peak of 2.4 mS/cm observed around Days 10-12, likely reflecting the release of soluble mineral ions during peak thermophilic decomposition activity. Following this peak, values declined slightly and stabilized around 2.1-2.2 mS/cm for the remainder of the process, including throughout the maturation phase (Days 45-61), indicating that ionic equilibrium was achieved relatively early and maintained consistently.

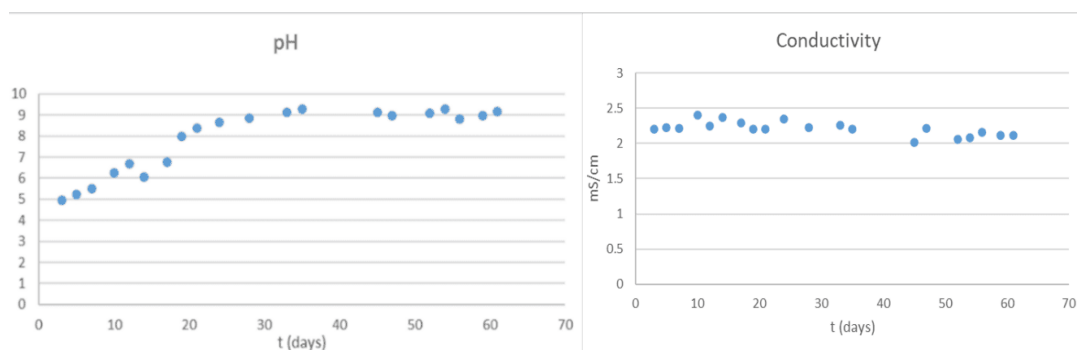


Figure 90. pH evolution in Compost Pile 3 over the composting period.

8. Conclusions

The results presented in Deliverable D4.4 demonstrate that the SYMSITES approach constitutes a technically robust and versatile solution for the valorisation of urban, industrial, and organic waste streams into high value new resources (HVNR). The integration of different treatment and conversion technologies, including AnMBR, pyrolysis, and membrane processes, has proven effective in generating multiple valuable outputs such as renewable energy carriers, reclaimed water, biofertilisers, and platform chemicals.

The validation activities confirm the technical feasibility of energy recovery pathways, with significant methane production and its subsequent conversion into hydrogen, contributing to improved energy efficiency and potential self-sufficiency of wastewater treatment plants. In parallel, the anaerobic sludge produced within the system exhibits favourable agronomic properties and complies with current regulatory thresholds for heavy metals, supporting its use as soil amendment, provided that appropriate hygienisation steps are implemented to ensure microbiological safety.

The assessment of reclaimed water highlights its strong potential for reuse in urban and agricultural applications, achieving high quality standards in most cases. However, the variability observed in parameters such as turbidity and suspended solids indicates the need for further optimisation to ensure consistent compliance with stricter quality classes and to expand its applicability in more demanding uses.

The industrial validation carried out, particularly in the textile sector, confirms that treated wastewater can successfully replace conventional water without compromising product quality, as demonstrated by minimal colour deviations in dyeing processes. This represents a significant step towards reducing freshwater consumption in industrial operations and enhancing circular resource use.

Additionally, the recovery of platform molecules such as benzoic acid, together with the production of biochar and bio-based products, further demonstrates the capacity of the system to generate added value beyond conventional waste treatment, fostering new market opportunities and industrial symbiosis.

Despite these positive outcomes, the deliverable also identifies several challenges that must be addressed for large-scale implementation. These include process stability and optimisation, energy consumption in certain configurations, operational issues such as clogging in irrigation systems, and regulatory constraints that may limit the use of some recovered products in specific regions.

In conclusion, D4.4 provides strong evidence that the SYMSITES concept is a viable and promising solution for advancing circular economy strategies through the recovery and reuse of resources from waste streams. While further optimisation and alignment with regulatory frameworks are required, the results clearly demonstrate the potential for real-world deployment and significant environmental, economic, and industrial benefits.





SYMSITES

High Value New Resources validation

Deliverable name: High Value New Resources validation	
ANNEX	D4.4_ANNEX I_AITEX (a) Description of equipment and materials

Content

1	Equipment used for the dyeing processes	2
2	Ingredients and materials.....	3
3	Dyeing curves applied.....	5



1 Equipment used for the dyeing processes

The equipment used to carry out the dyeing process are the Pyrotec 4 (for the fabric pre-industrial scaleup) by Roaches, an industrial rotative washing machine (for the fabric industrial scaleup) and Loris Bellini RBNV 270 (for the industrial yarn scaleup). All three equipments are shown in the Figures below.



Figure 1. Pyrotec 4 by Roaches



Figure 2. Pre-industrial rotative washing machine



Figure 3. Loris Bellini RBNV 270



2 Ingredients and materials

WASTEWATER

The waters used to carry out the dyeing process are the following:

1. 100% process water (reference)
2. 85% process water + 15% WW from Spanish Ecosite pilot plant
3. 75% process water + 25% WW from Spanish Ecosite pilot plant

TEXTILE SUBSTRATES

The textiles substrates used in for the dyeing process are the following:

- Cotton openwork fabric (weight 180 g/m²)
- Polyester openwork fabric (weight 145 g/m²)
- Acrylic fabric (weight 313 g/m²)
- Wool fabric (weight 226 g/m²)
- Polyester yarn

DYES

Pre-industrial (Protec 4).

Direct dyes

- Unitary color: Blue 4BL (0,875 g)
- Trichromy: Blue 4BL (0,2197 g) + Yellow TL (0,2197 g) + Red 6B (0,2197 g)

Disperse dyes

- Unitary color: Asuperse Azul P-FBL 160 (0,875 g)
- Trichromy: Asuperse Azul P-FBL 160 (0,2625 g) + Asuperse Amarillo P-FBL (0,5250 g) + Asuperse Rojo P-FBL 200 (0,0875 g)

Industrial (Rotative washing machine).

Direct dyes

- Unitary color: Blue 4BL (5 g)
- Trichromy: Blue 4BL (1,667 g) + Yellow TL (1,667 g) + Red 6B (1,667 g)

Acid dyes

- Unitary color: Azul Nylacid BLN (3g)
- Trichromy: Azul Nylacid BLN (1g) + Amarillo Nylacid 3RL (1g) + Rojo Nyalacid 2RBL (1g)

Basic dyes

- Unitary color: Azul Astrazón FGRL (3g)
- Trichromy: Azul Astrazón FGRL (1g) + Amarillo Oro Hispacril GL 200 (2g) + Rojo Astrazón FGRL (2g)

Industrial dyeing yarns.

Disperse dyes

- Unitary color: Dianix Rubine (12,687g)
- Trichromy: Dianix Yellow (16,8303g), Dianix Blue (7,2130g) and Dianix Rubine (1,7174g)

The table below summarizes the different experiments carried out as well as the different technologies applied to characterize the samples.



Table 1. Scaleup experiments carried out

Scale	Kind of Dye	Material	Colour	% Process water	Samples dyed	Assessment
Scaleup pre-industrial dyeing (Tint Control)	Disperse	Polyester Fabric	Unitary	100% (reference)	1x	COLORIMETRY (3 measurement areas, 5rep/area)
				85% (15% PP WW)	1x	
				75% (25% PP WW)	1x	
			Trichromy	100% (reference)	1x	
				85% (15% PP WW)	1x	
				75% (25% PP WW)	x	
	Direct	Cotton Fabric	Unitary	100% (reference)	1x	
				85% (15% PP WW)	1x	
				75% (25% PP WW)	1x	
			Trichromy	100% (reference)	1x	
				85% (15% PP WW)	1x	
				75% (25% PP WW)	1x	
Scaleup industrial dyeing - rotative washing machine	Direct	Cotton Fabric	Unitary	100% (reference)	1x	WASHING FASTNESS (1 replicate/sample)
				85% (15% PP WW)	1x	
				75% (25% PP WW)	1x	
			Trichromy	100% (reference)	1x	
				85% (15% PP WW)	1x	
				75% (25% PP WW)	1x	
	Basic	Acrylic Fabric	Unitary	100% (reference)	1x	
				85% (15% PP WW)	1x	
				75% (25% PP WW)	1x	
			Trichromy	100% (reference)	1x	
				85% (15% PP WW)	1x	
				75% (25% PP WW)	1x	
	Acid	Wool Fabric	Unitary	100% (reference)	1x	
				85% (15% PP WW)	1x	
				75% (25% PP WW)	1x	
			Trichromy	100% (reference)	1x	

Scale	Kind of Dye	Material	Colour	% Process water	Samples dyed	Assessment
Scaleup industrial dyeing – Package dyeing	Disperse	PES Yarn	Unitary	85% (15% PP WW)	1x	
				75% (25% PP WW)	1x	
				100% (reference)	1x	
				85% (15% PP WW)	1x	
				75% (25% PP WW)	1x	
				100% (reference)	1x	
			Trichromy	85% (15% PP WW)	1x	
				75% (25% PP WW)	1x	
				100% (reference)	1x	

3 Dyeing curves applied

Cotton dyeing

Direct dyes are going to be used for the cotton dyeing procedure. The dyeing curves used can be seen in the Figure 4 and 5. In Figure 4 it can be seen the pre-industrial scaleup done in Pyrotec 4. In Figure 5 it can be seen the industrial scaleup done in the rotative washing machine.

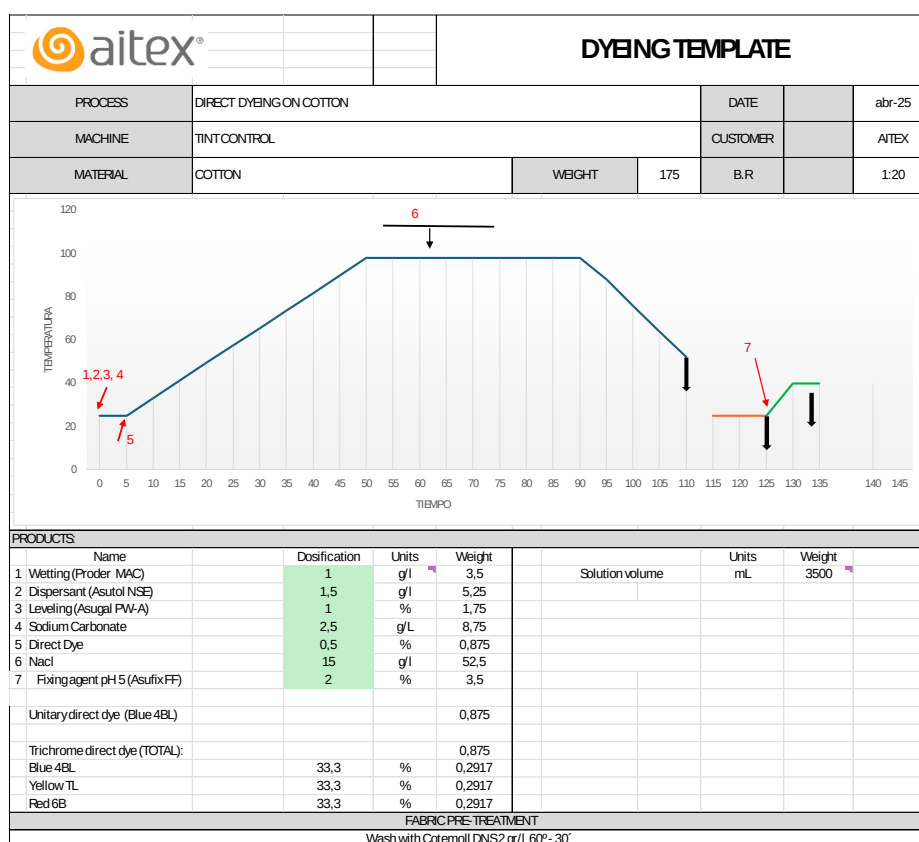


Figure 4. Scaleup pre-industrial cotton dyeing curve

At first, the cotton fabric is pre-treated by washing it with Cotemoll DNS (2 g/L) at 60°C for 30 minutes.

The bath ratio (or liquor ratio) of this dye is 1:20, and the weight of the fabric sample is 175 g.

To make the dye bath, the auxiliary products (wetting (1 g/L), dispersant (1,5 g/L), leveling (1 g/L) and sodium carbonate (2,5 g/L)) are first mixed in water. Once the auxiliary products are mixed, the dye (0,5 % w.o.f) is added to the mixture. Finally, after approximately 1 hour, NaCl (15g/L) is added

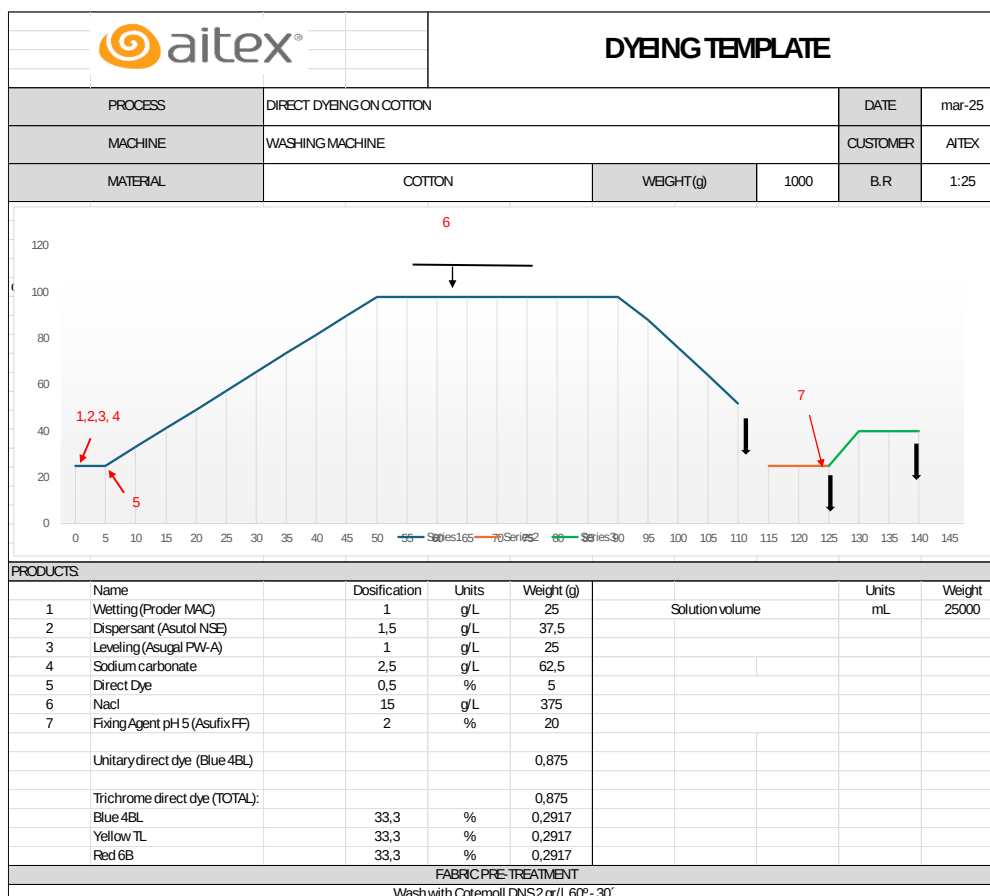


Figure 5. Scaleup industrial cotton dyeing curve

The bath ratio (or liquor ratio) of this dye is 1:25, and the weight of the fabric sample is 1000 g.

To make the dye bath, the auxiliary products (wetting (1 g/L), dispersant (1,5 g/L), leveling (1 g/L) and sodium carbonate (2,5 g/L)) are first mixed in water. Once the auxiliary products are mixed, the dye (0,5 % w.o.f) is added to the mixture. Finally, after approximately 1 hour, NaCl (15g/L) is added.

Polyester dyeing

Disperse dyes are going to be used for the polyester dyeing procedure. The dyeing curve of polyester dyeing can be seen in the Figure 6.

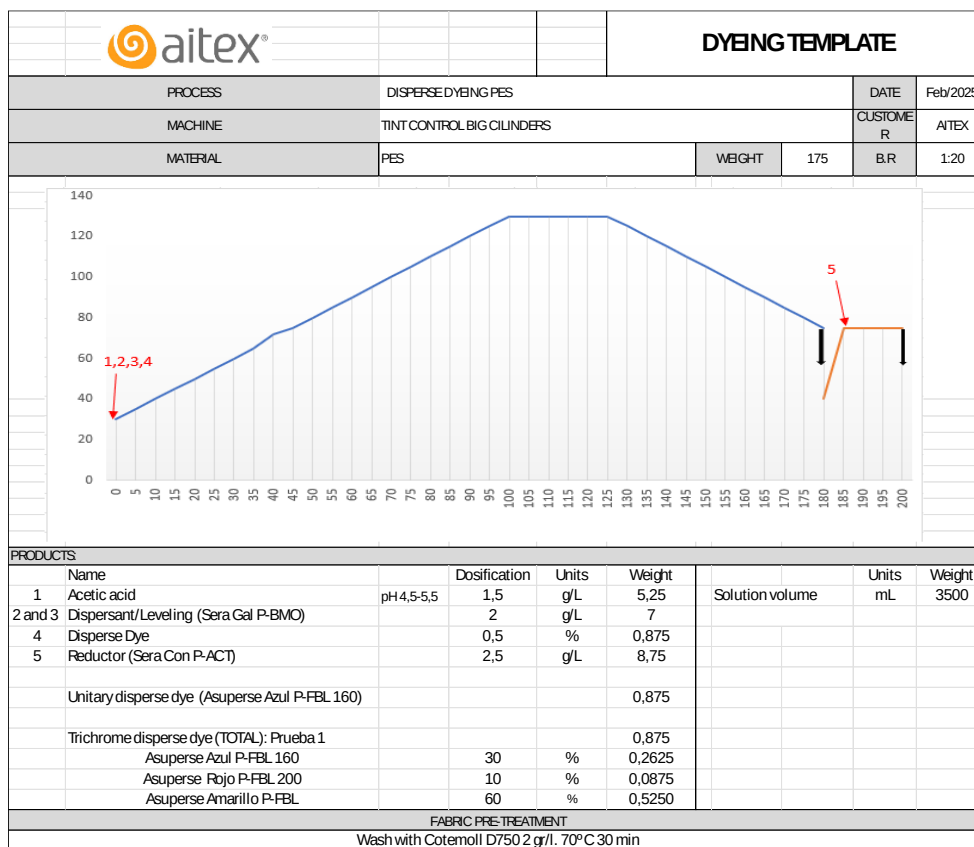


Figure 6. Polyester dyeing curve

At first, the polyester fabric is pre-treated by washing it with Cotemoll D750 (2 g/L) at 70°C for 30 minutes.

The bath ratio (or liquor ratio) of this dye is 1:20, and the weight of the fabric is 175 g.

To make the dye bath, the auxiliary products (acetic acid (1,5 g/L), levelling (1 g/L), and dispersant (1 g/L)) are first mixed in water. Once the auxiliary products are mixed, the dye (0,5 % w.o.f) is added to the mixture. Finally, after approximately 45 minutes, the reductor agent is added.

Acrylic dyeing

Basic dyes are going to be used for the acrylic dyeing procedure. The dyeing curve of the acrylic dyeing can be seen in Figure 7.



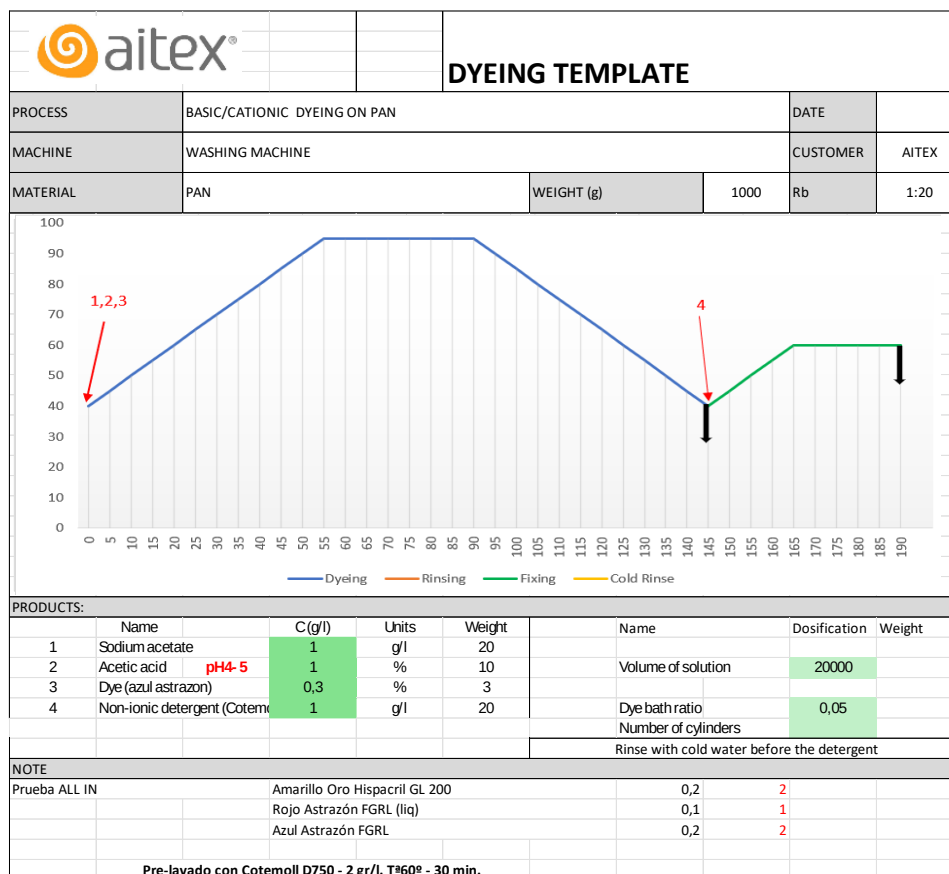


Figure 7. Acrylic dyeing curve

At first, the acrylic fabric is pre-treated by washing it with Cotemoll D750 (2 g/L) at 60°C for 30 minutes.

In the acrylic dyeing procedure, the sodium acetate (1 g/L), the acetic acid (1 % w.o.f) and the dye (1 % w.o.f) are added together in the dye bath at 40°C. Then when the temperature rises and arrives to 95°C, it stays for 35 minutes and then decreases until 40°C.

Once the dyeing process is finished a non-ionic detergent (1 g/L) is used for finishing the procedure.

Wool dyeing

Acid dyes are going to be used for the wool dyeing procedure. The dyeing curve of the wool dyeing can be seen in Figure 8.



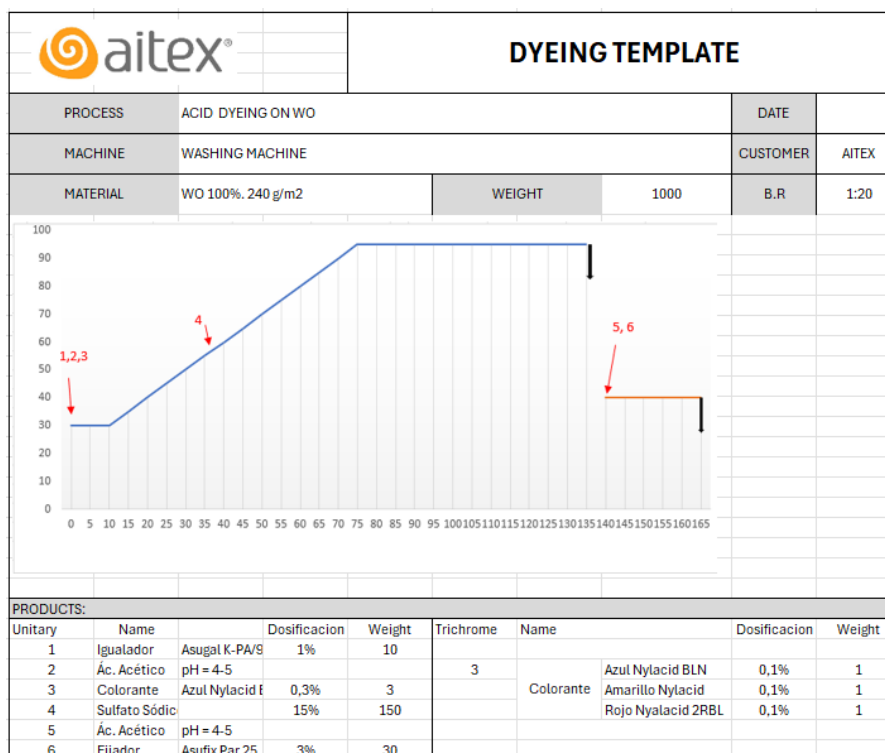


Figure 8. Wool dyeing curve

In the wool dyeing procedure, the levelling (1% w.o.f), the acetic acid and the dye (0,3% w.o.f) are added together in the dye bath at 30°C. Then when the temperature arrives to 60°C the sodium sulfate (15% w.o.f) is added, and the keeps raising until it reaches 90°C where it stays 60 minutes.

Once the dyeing process is finished the fixing agent (3% w.o.f), and acetic acid are added to fix the dye.

Polyester yarn dyeing

First, PES yarns provided by JOVER are backwinded onto perforated plastic bobbins for dyeing, which are specifically designed to ensure uniform liquor flow during subsequent wet processing. This step is critical, as the package build quality directly influences dye penetration, levelness, and overall process reproducibility. Key parameters such as winding tension, package density, yarn alignment, and final package weight must be carefully controlled and adjusted to avoid defects such as channeling, uneven dye uptake, or yarn deformation.

Once the bobbins are done, preparation of the fibers is the first step in the processing. Recipe for preparation curve for this preparation in **Figure 9**. Shortly, a wetting-deoiling agent (1,5 g/L) is added with sodium carbonate (1 g/L) to help increase pH and temperature rise to 80 °C for 20 min.

	Recipe for Customer trial	Test N°
--	----------------------------------	----------------

Recipe code SYMSITES	Shade Prep.	Customer AITEX	Dyeing program 200
Machine N° RBNV 270/975 IN 16120	Date Art.	Lot weight Kg. 1,921	Machine litres 55
			N° of bobbins 3
			Machine level / cm 7 / 57

Product / Dyestuff	%	gr/lt	Quantity (grams)
Introd. Vaso Dyeing			
1 1 PRODER RK MOD 85		1,5	82,5
2 1 Carbonato Sodico		1,0	55,0

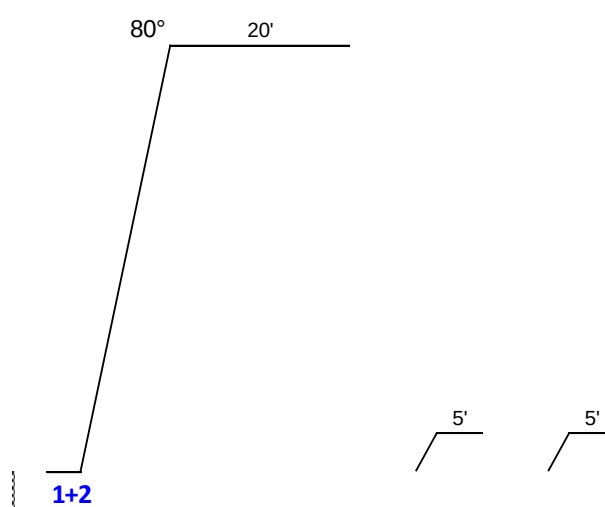


Figure 9. Dyeing recipe and curve for preparation of PES yarn

For dyeing steps, the following recipe and curve are followed. Briefly, dyeing auxiliaries and acetic acid (1,5 g/L) are added to the dyeing machine. Then, colour (1,5 % w.o.f) is added and temperature is rise to 130 °C in different steps to assure adsorption equilibria is achieved. After dyeing, a reductive rinse is needed to remove all the colour that has not penetrated into the fibre.





Recipe code SYMSITEX	Shade Unitary	Customer AITEX		Dyeing program 215 paso 12
Machine N° RBNV 270/975 IN 16120	Date Art.	Lot weight Kg. 0,846	Machine litres 40	N° of bobbins 1
				Machine level / cm 6 / 57

Product / Dyestuff	%	gr/lt	Quantity (gramms)	
3 1 Asutol I-222		1,0	40,0	
3 1 Ac. Acetico		0,3	12,0	
				ph= 5
Dyestuff				
4 Dianix Rubine	1,5000		12,687	
Stripping				
4 1 Caustic Soda		1,5	60,0	
4 1 Sodyum Hidrosulphite		3	120,0	
5 1 Acetic Acid		1,5	60,0	
Finishing				
6 1 Acetic Acid		0,3	12,0	

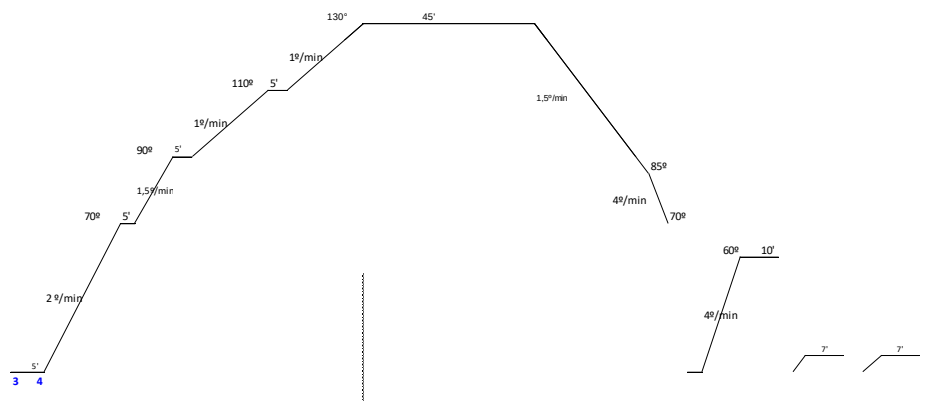


Figure 10. Dyeing recipe and curve for polyester dyeing





SYMSITES

High Value New Resources validation

Deliverable name: High Value New Resources validation	
ANNEX	D4.4_ANNEX I_AITEX (b) Fabrics dyed and Boxplots with L*, a* and b* results

Content

1	Image of the dyed samples	2
2	BOXPLOTS WITH L, A AND B COORDINATES	12



1 Image of the dyed samples

Below are shown different tables with the results of the dyed samples using the different nature textile substrates and the different percentages of wastewater treated by the Spanish Ecosite Pilot Plant.

PRE-INDUSTRIAL SCALEUP

Table 1. Images of the pre-industrial scaleup of unitary polyester dyeing for 100%, 85% and 75% of process water

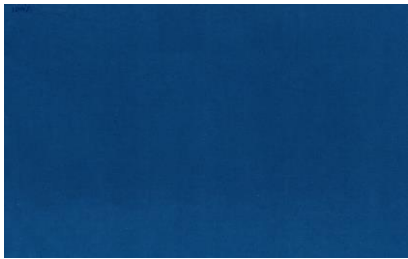
EXPERIMENT	WATER	IMAGE OF THE DYED SAMPLE
PRE-INDUSTRIAL SCALEUP POLYESTER UNITARY DYEING	100% PW (reference)	
	85% PW (15% PP WW)	
	75% PW (25% PP WW)	



Table 2. Images of the pre-industrial scaleup of trichromy polyester dyeing for 100%, 85% and 75% of process water

EXPERIMENT	WATER	IMAGE OF THE DYED SAMPLE
PRE-INDUSTRIAL SCALEUP POLYESTER TRICHROMY DYEING	100% PW (reference)	
	85% PW (15% PP WW)	
	75% PW (25% PP WW)	

Table 3. Images of the pre-industrial scaleup of unitary cotton dyeing for 100%, 85% and 75% of process water

EXPERIMENT	WATER	IMAGE OF THE DYED SAMPLE
PRE-INDUSTRIAL SCALEUP COTTON UNITARY DYEING	100% PW (reference)	





EXPERIMENT	WATER	IMAGE OF THE DYED SAMPLE
	85% PW (15% PP WW)	
	75% PW (25% PP WW)	

Table 4. Images of the pre-industrial scaleup trichromy cotton dyeing for 100%, 85% and 75% of process water

EXPERIMENT	WATER	IMAGE OF THE DYED SAMPLE
PRE-INDUSTRIAL SCALEUP COTTON TRICHROMY DYEING	100% PW (reference)	
	85% PW (15% PP WW)	





EXPERIMENT	WATER	IMAGE OF THE DYED SAMPLE
	75% PW (25% PP WW)	

INDUSTRIAL SCALEUP

Table 5. Images of the industrial scaleup of unitary cotton dyeing for 100%, 85% and 75% of process water

EXPERIMENT	WATER	IMAGE OF THE DYED SAMPLE
INDUSTRIAL SCALEUP COTTON UNITARY DYEING	100% PW (reference)	
	85% PW (15% PP WW)	
	75% PW (25% PP WW)	





Table 6. Images of the industrial scaleup of trichromy cotton dyeing for 100%, 85% and 75% of process water

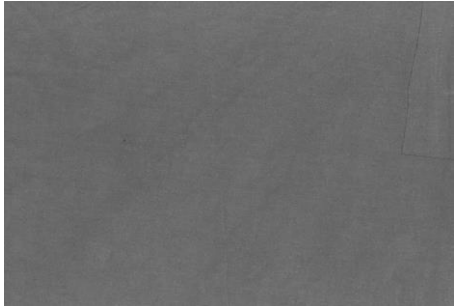
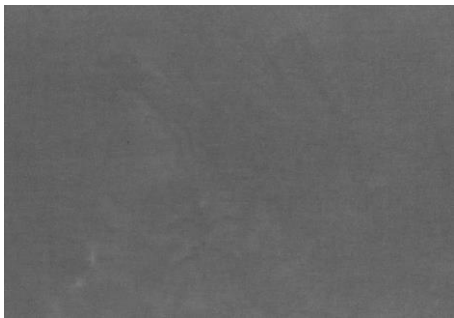
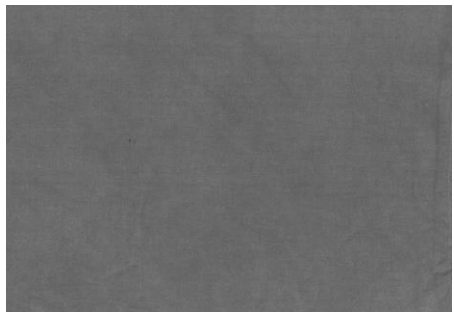
EXPERIMENT	WATER	IMAGE OF THE DYED SAMPLE
INDUSTRIAL SCALEUP COTTON TRICHROMY DYEING	100% PW (reference)	
	85% PW (15% PP WW)	
	75% PW (25% PP WW)	



Table 7. Images of the industrial scaleup of wool unitary dyeing for 100%, 85% and 75% of process water

EXPERIMENT	WATER	IMAGE OF THE DYED SAMPLE
INDUSTRIAL SCALEUP WOOL UNITARY DYEING	100% PW (reference)	
	85% PW (15% PP WW)	
	75% PW (25% PP WW)	

Table 8. Images of the industrial scaleup of wool trichromy dyeing for 100%, 85% and 75% of process water

EXPERIMENT	WATER	IMAGE OF THE DYED SAMPLE
INDUSTRIAL SCALEUP WOOL TRICHRAMY DYEING	100% PW (reference)	





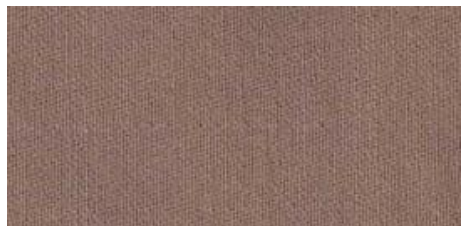
EXPERIMENT	WATER	IMAGE OF THE DYED SAMPLE
	85% PW (15% PP WW)	
	75% PW (25% PP WW)	

Table 9. Images of the industrial scaleup of acrylic unitary dyeing for 100%, 85% and 75% of process water

EXPERIMENT	WATER	IMAGE OF THE DYED SAMPLE
INDUSTRIAL SCALEUP ACRYLIC UNITARY DYEING	100% PW (reference)	
	85% PW (15% PP WW)	



EXPERIMENT	WATER	IMAGE OF THE DYED SAMPLE
	75% PW (25% PP WW)	

Table 10. Images of the industrial scaleup of acrylic trichromy dyeing for 100%, 85% and 75% of process water

EXPERIMENT	WATER	IMAGE OF THE DYED SAMPLE
INDUSTRIAL SCALEUP ACRYLIC TRICROMY DYEING	100% PW (reference)	
	85% PW (15% PP WW)	
	75% PW (25% PP WW)	



Table 11. Images of the industrial scaleup of polyester unitary dyeing for 100%, 85% and 75% of process water

EXPERIMENT	WATER	IMAGE OF THE DYED SAMPLE
INDUSTRIAL SCALEUP POLYESTER UNITARY DYEING	100% PW (reference)	
	85% PW (15% PP WW)	
	75% PW (25% PP WW)	



Table 12. Images of the industrial scaleup of polyester trichromy dyeing for 100%, 85% and 75% of process water

EXPERIMENT	WATER	IMAGE OF THE DYED SAMPLE
INDUSTRIAL SCALEUP POLYESTER TRICHROMY DYEING	100% PW (reference)	
	85% PW (15% PP WW)	
	75% PW (25% PP WW)	

In general, and at first glance based on the results shown in the tables above, there is no significant visible difference between the samples dyed with pure water (reference) and those dyed with wastewater treated by the Spanish Ecosite pilot plant.



2 BOXPLOTS WITH L, A AND B COORDINATES

The following Figures show in boxplots the colorimetric results of the coordinates L^* , a^* and b^* obtained in the dyeing results of the different textiles substrates.

- PRE-INDUSTRIAL SCALEUP

Unitary blue over polyester Fabric

The boxplots below show the L^* , a^* and b^* values obtained from the colourimeter for the 3 experiments: 100% process water (reference), 85% (15% WW from Pilot Plant) and 75% (25% WW from Pilot Plant).

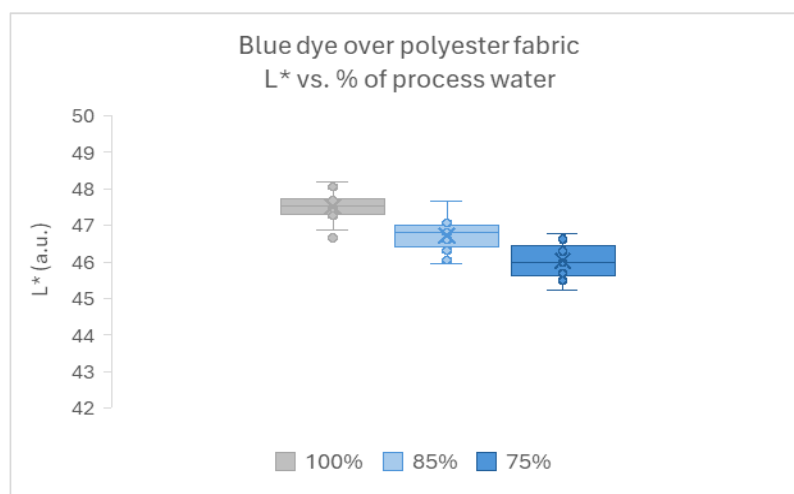


Figure 1. Boxplot for Blue dye over Polyester fabric: L^* vs. % Process water

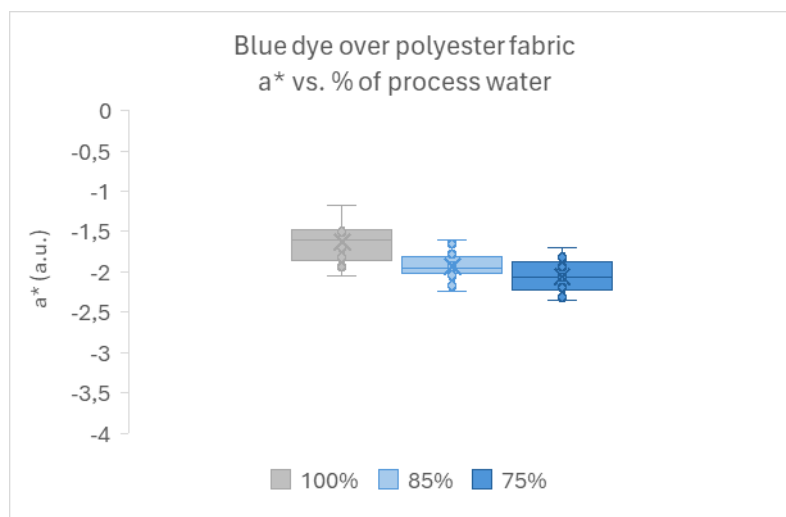


Figure 2. Boxplot for Blue dye over Polyester fabric: a^* vs. % Process water



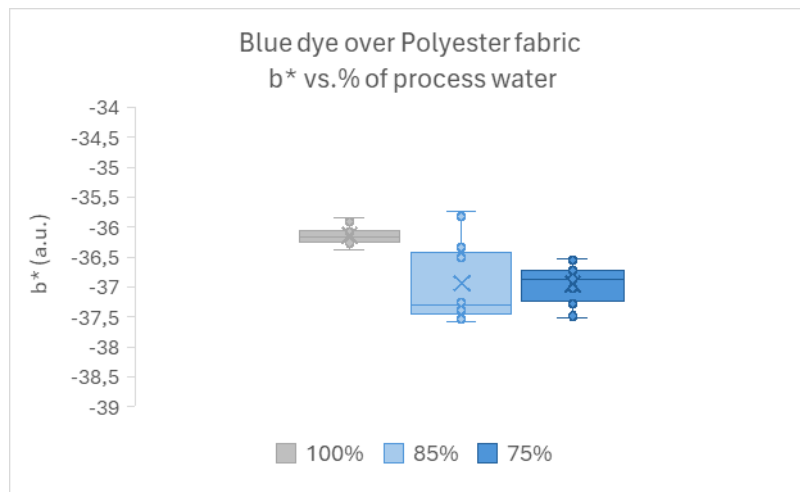


Figure 3. Boxplot for Blue dye over Polyester fabric: b* vs. % Process water

Trichromy over polyester fabric

The boxplots below show the L*, a* and b* values obtained from the colourimeter for the 3 experiments: 100% process water (reference), 85% (15% WW from Pilot Plant) and 75% (25% WW from Pilot Plant).

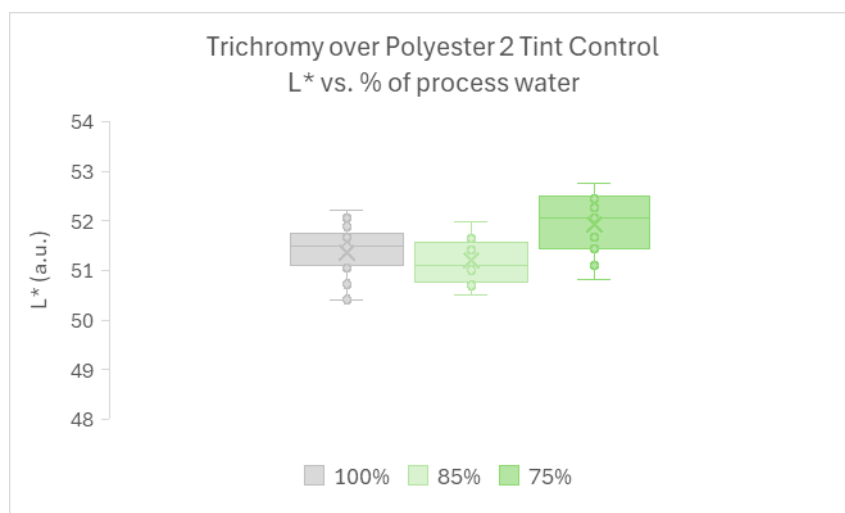


Figure 4. Boxplot for trichromy over Polyester fabric: L* vs. % Process water

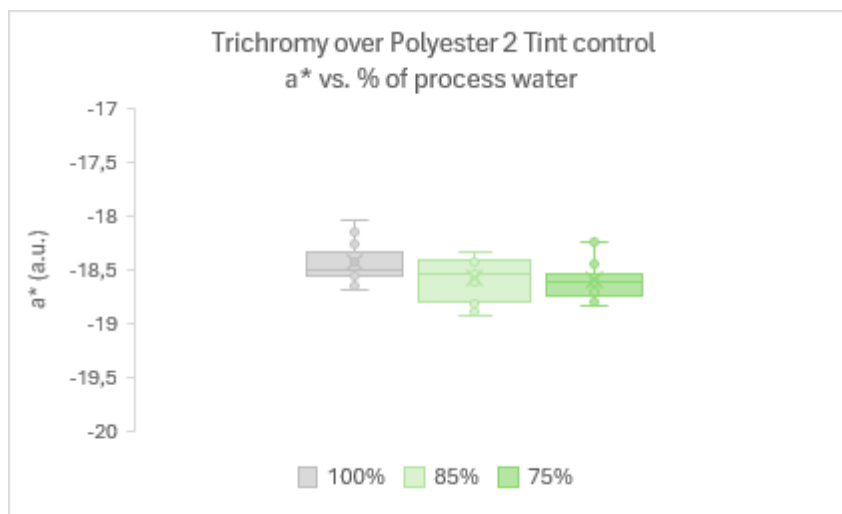


Figure 5. Boxplot for trichromy over Polyester fabric: a* vs. % Process water

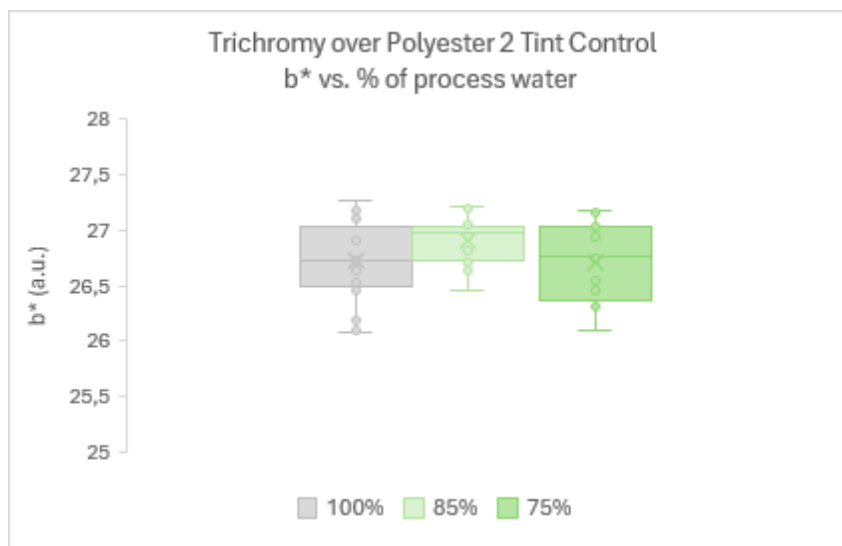


Figure 6. Boxplot for trichromy over Polyester fabric: b* vs. % Process water

Unitary dye over cotton fabric

The boxplots below show the L*, a* and b* values obtained from the colourimeter for the 3 experiments: 100% process water (reference), 85% (15% WW from Pilot Plant) and 75% (25% WW from Pilot Plant).



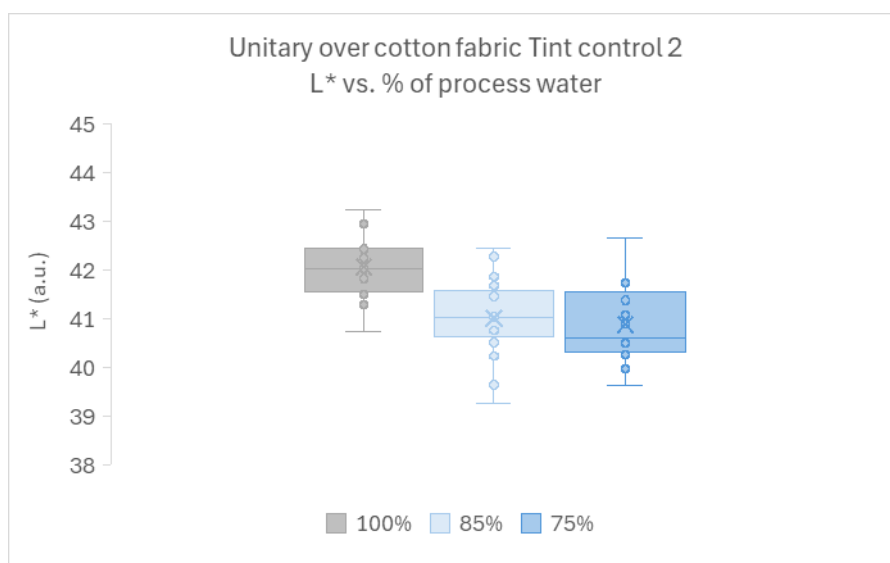


Figure 7. Boxplot for unitary over Cotton fabric: L* vs. % Process water

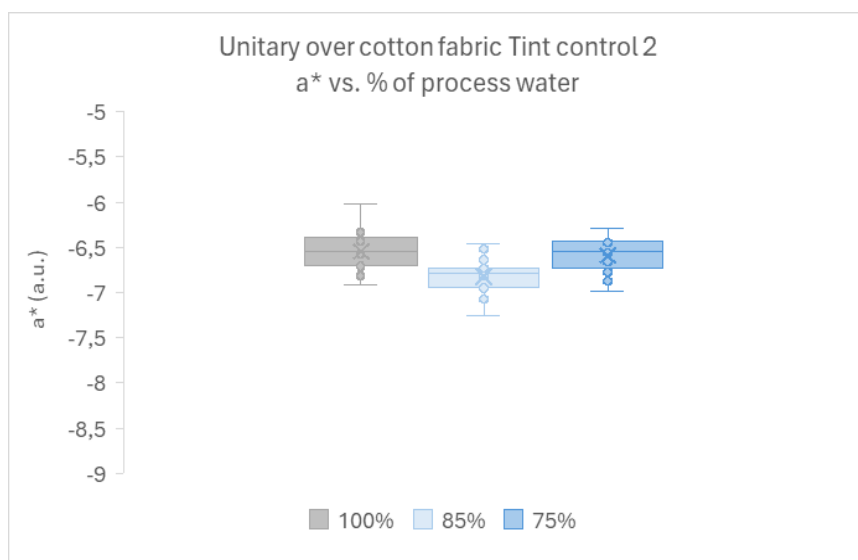


Figure 8. Boxplot for unitary over Cotton fabric: a* vs. % Process water



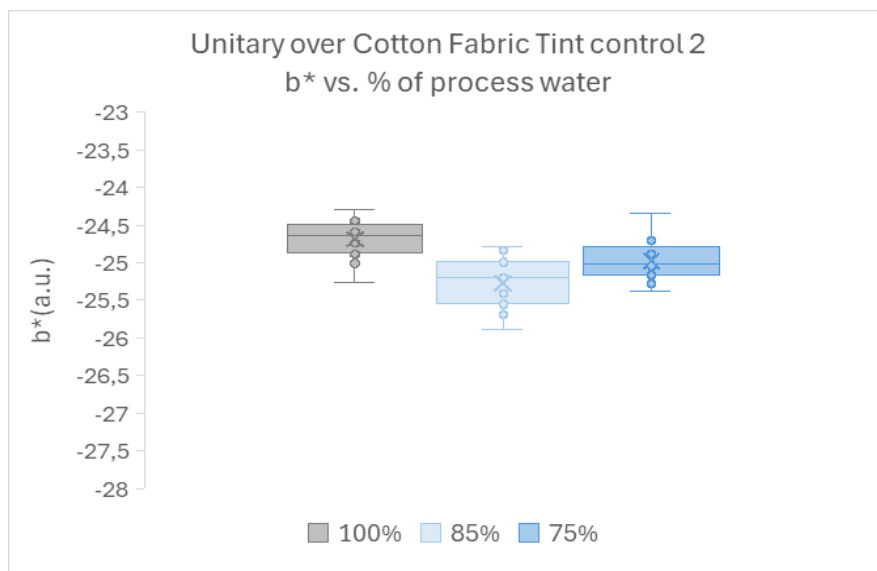


Figure 9. Boxplot for unitary over Cotton fabric: b* vs. % Process water

Trichromy over cotton fabric

The boxplots below show the L*, a* and b* values obtained from the colourimeter for the 3 experiments: 100% process water (reference), 85% (15% WW from Pilot Plant) and 75% (25% WW from Pilot Plant).

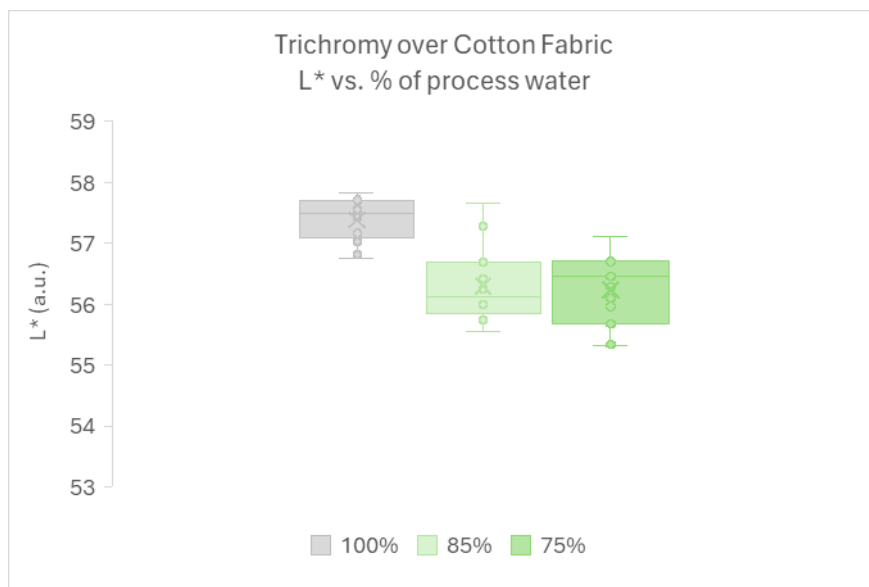


Figure 10. Boxplot for Trichromy over Cotton fabric: L* vs. % Process water



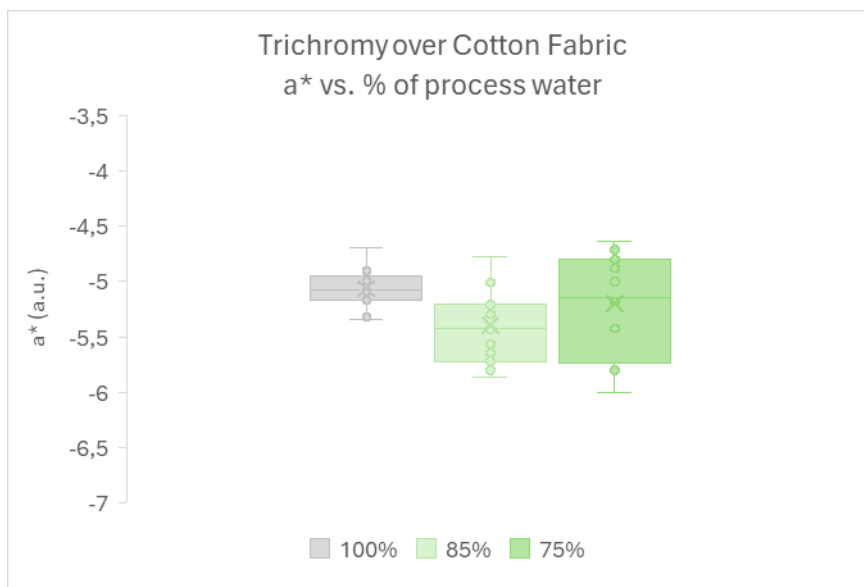


Figure 11. Boxplot for Trichromy over Cotton fabric: a^* vs. % Process water

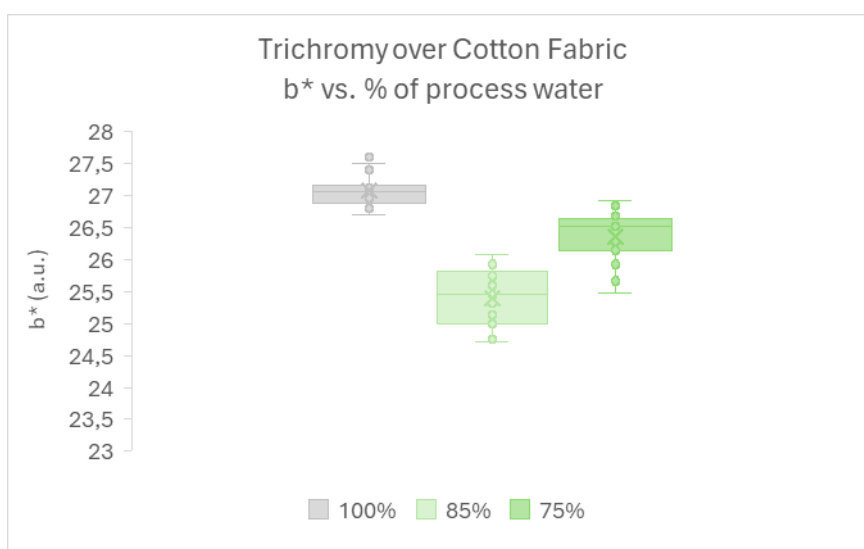


Figure 12. Boxplot for Trichromy over Cotton fabric: b^* vs. % Process water

- INDUSTRIAL SCALEUP

Unitary yellow over cotton fabric

The boxplots below show the L^* , a^* and b^* values obtained from the colourimeter for the 3 experiments: 100% process water (reference), 85% (15% WW from Pilot Plant) and 75% (25% WW from Pilot Plant).



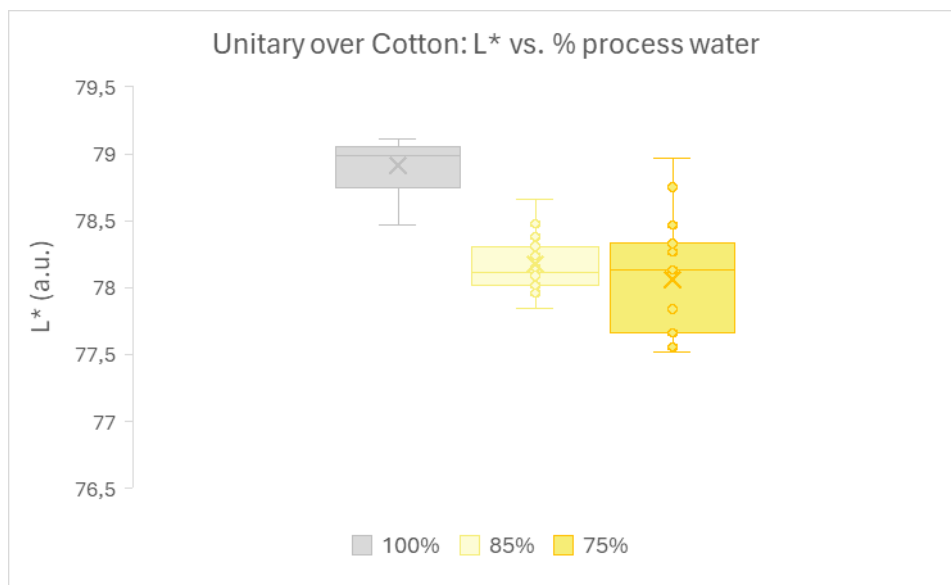


Figure 13. Boxplot for Unitary over Cotton fabric: L^* vs. % Process water

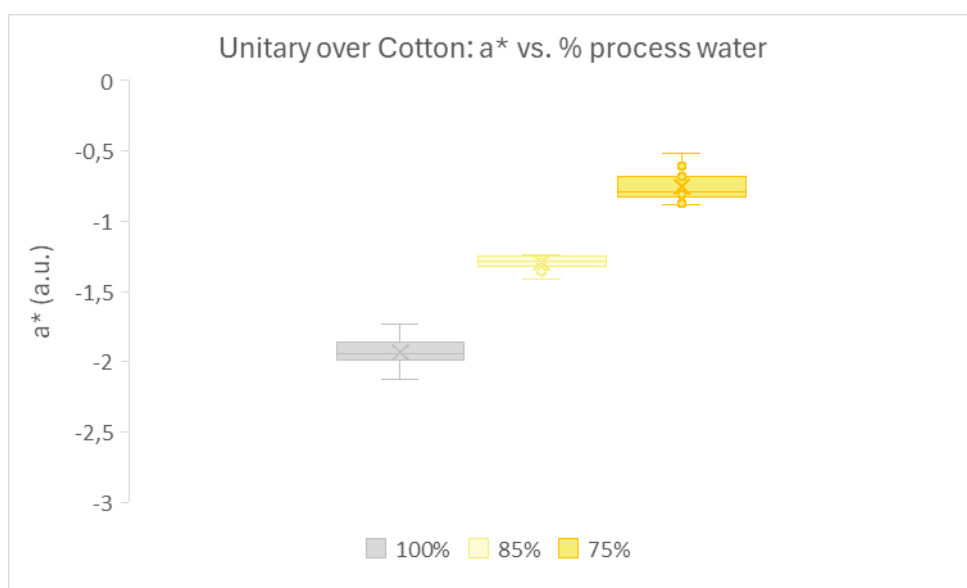


Figure 14. Boxplot for Unitary over Cotton fabric: a^* vs. % Process water



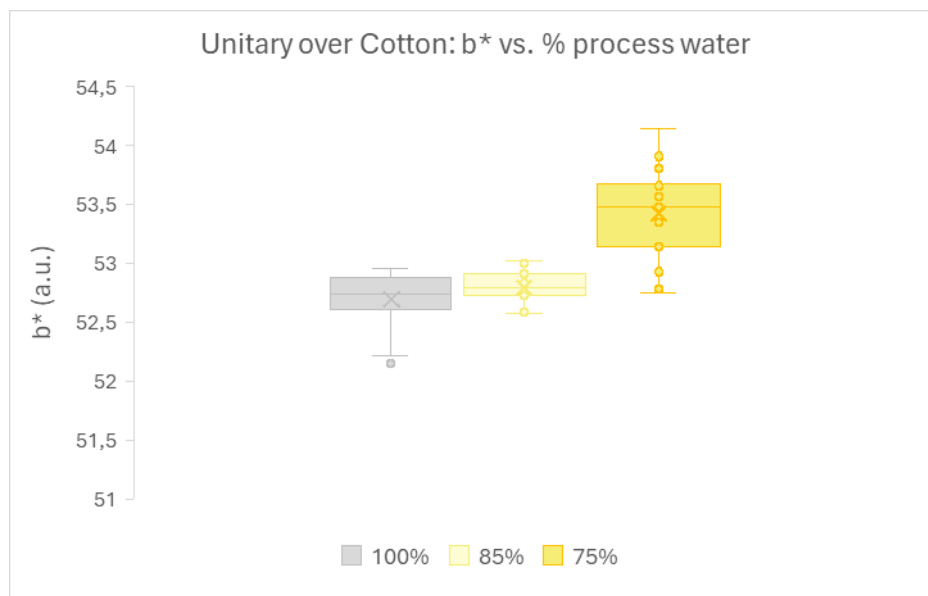


Figure 15. Boxplot for Trichromy over Cotton fabric: b^* vs. % Process water

Trichromy over cotton fabric

The boxplots below show the L^* , a^* and b^* values obtained from the colourimeter for the 3 experiments: 100% process water (reference), 85% (15% WW from Pilot Plant) and 75% (25% WW from Pilot Plant).

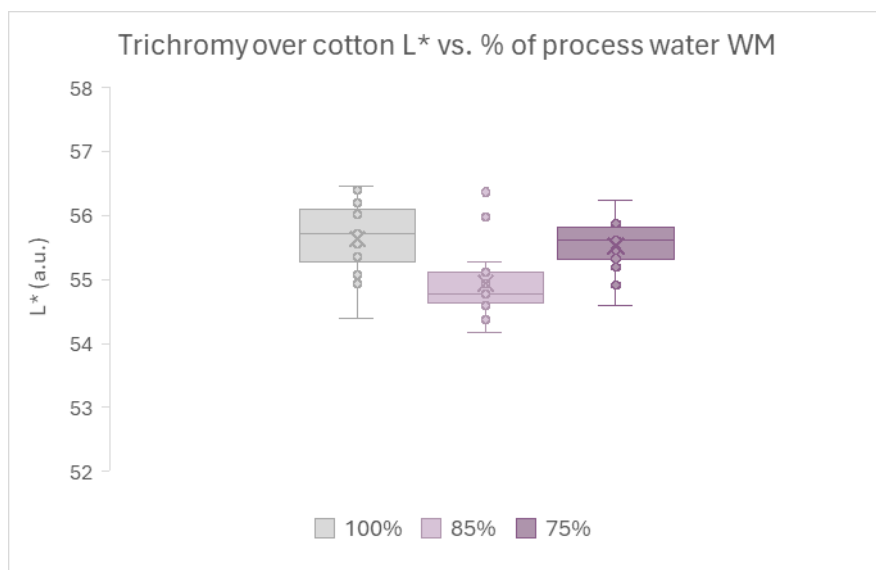


Figure 16. Boxplot for Trichromy over Cotton fabric: L^* vs. % Process water



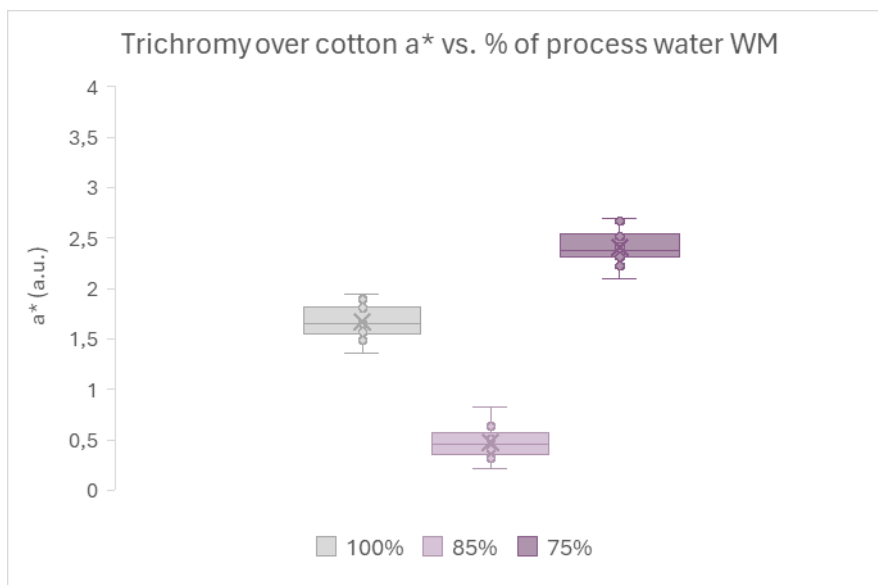


Figure 17. Boxplot for Trichromy over Cotton fabric: a* vs. % Process water

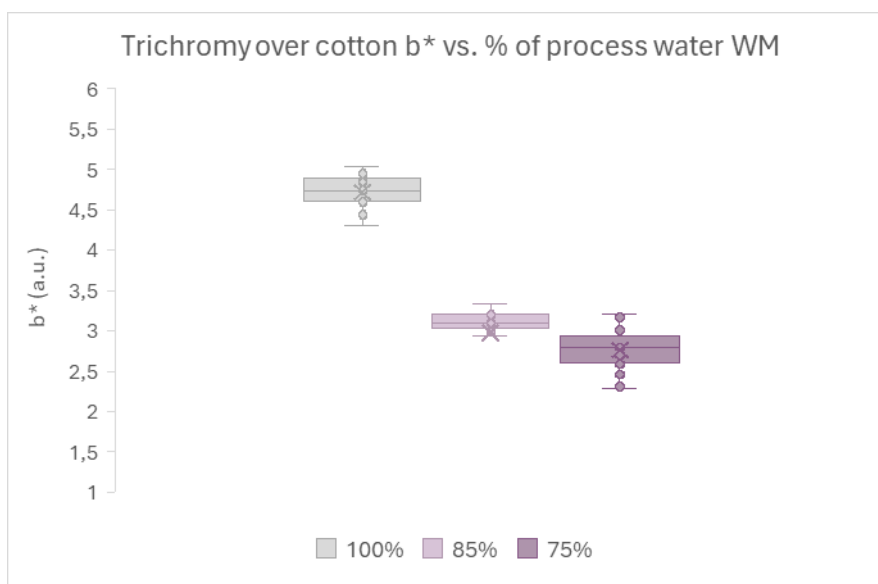


Figure 18. Boxplot for Trichromy over Cotton fabric: b* vs. % Process water

Unitary blue over acrylic fabric

The boxplots below show the L*, a* and b* values obtained from the colourimeter for the 3 experiments: 100% process water (reference), 85% (15% WW from Pilot Plant) and 75% (25% WW from Pilot Plant).



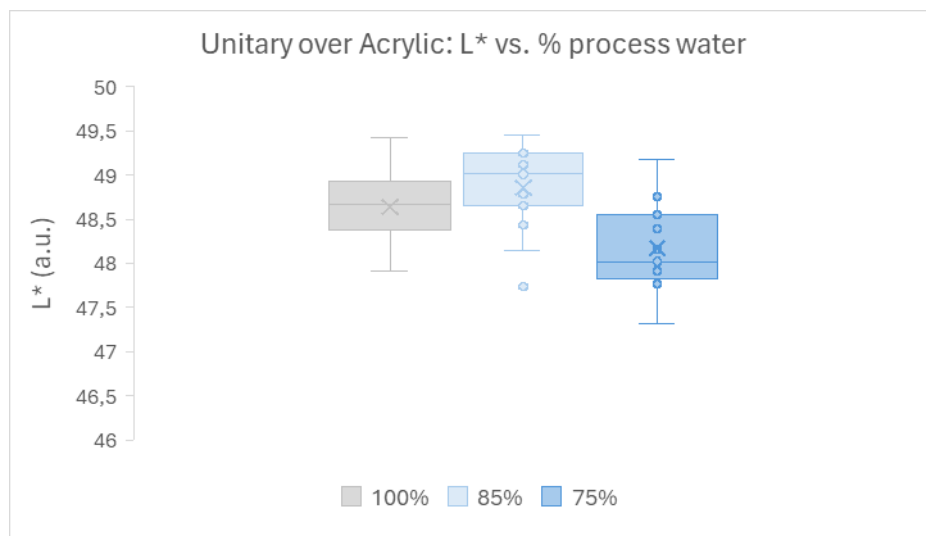


Figure 19. Boxplot for Unitary over Acrylic fabric: L^* vs. % Process water

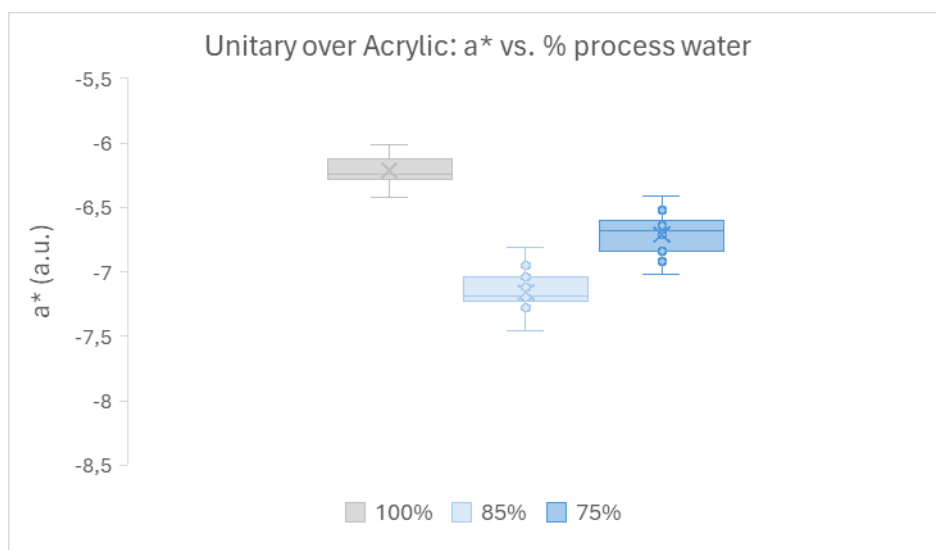


Figure 20. Boxplot for Unitary over Acrylic fabric: a^* vs. % Process water



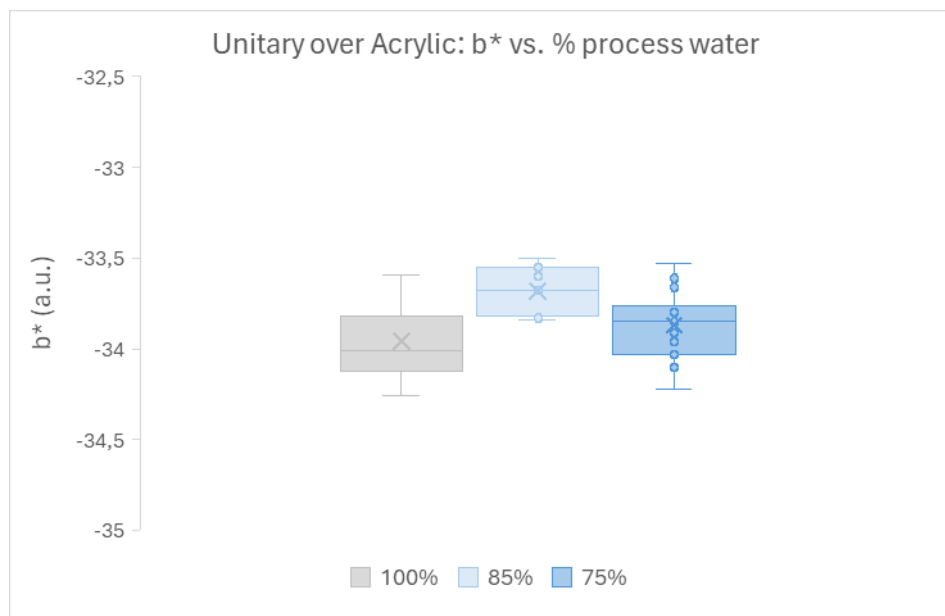


Figure 21. Boxplot for Unitary over Acrylic fabric: b^* vs. % Process water

Trichromy over acrylic fabric

The boxplots below show the L^* , a^* and b^* values obtained from the colourimeter for the 3 experiments: 100% process water (reference), 85% (15% WW from Pilot Plant) and 75% (25% WW from Pilot Plant).

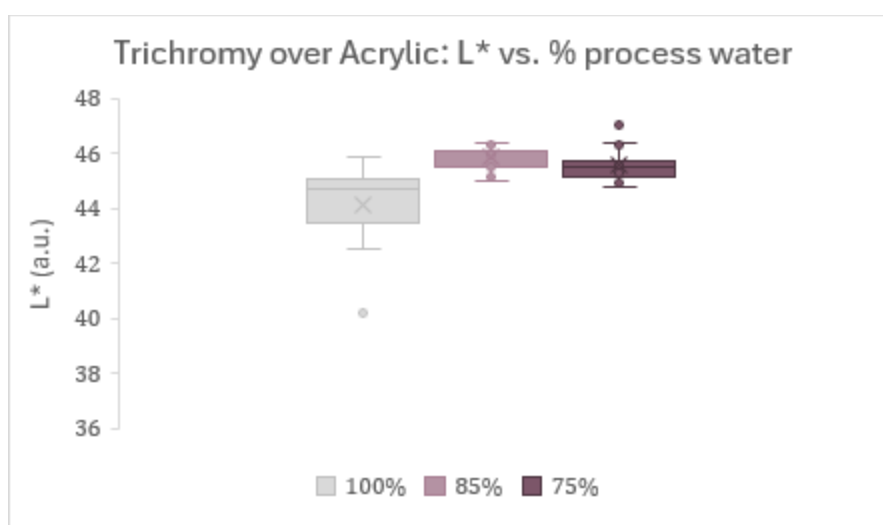


Figure 22. Boxplot for Trichromy over Acrylic fabric: L^* vs. % Process water



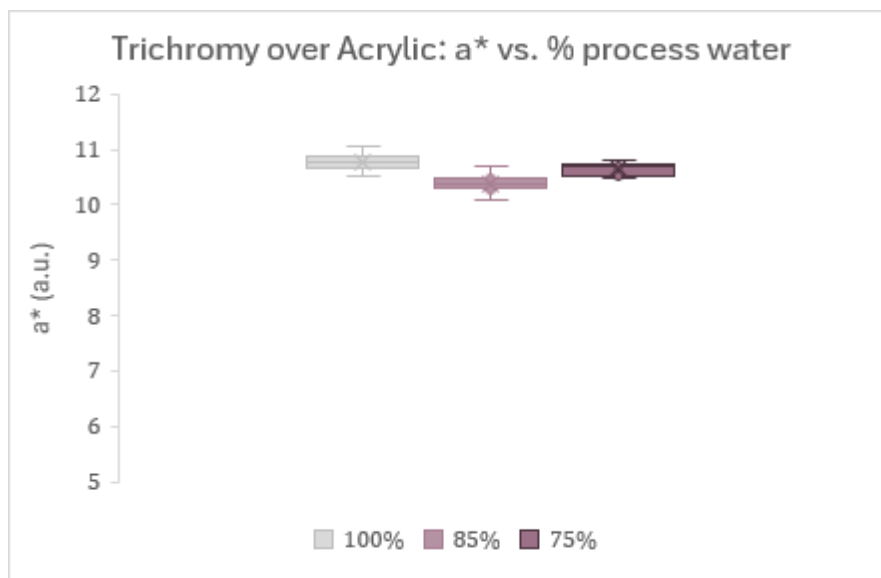


Figure 23. Boxplot for Trichromy over Acrylic fabric: a^* vs. % Process water

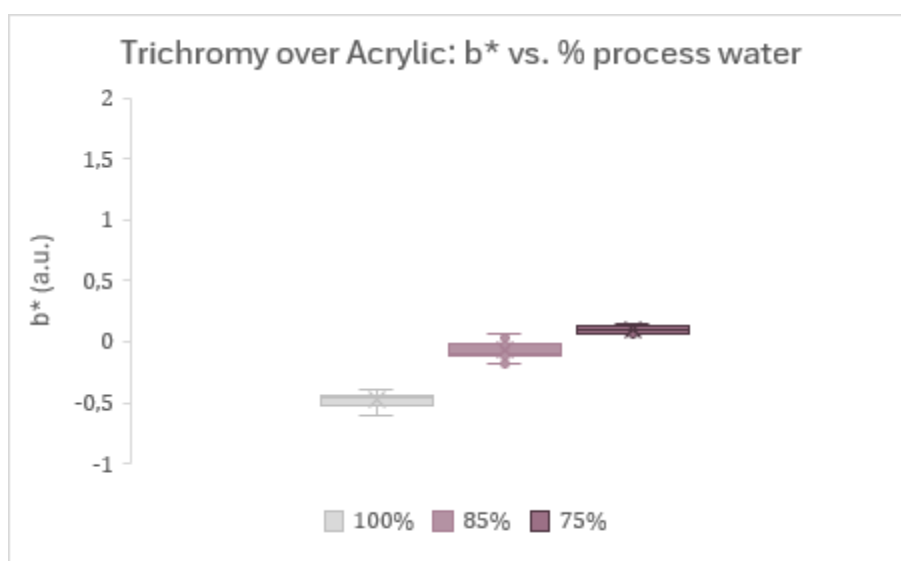


Figure 24. Boxplot for Trichromy over Acrylic fabric: b^* vs. % Process water

Unitary blue over Wool fabric

The boxplots below show the L^* , a^* and b^* values obtained from the colourimeter for the 3 experiments: 100% process water (reference), 85% (15% WW from Pilot Plant) and 75% (25% WW from Pilot Plant).



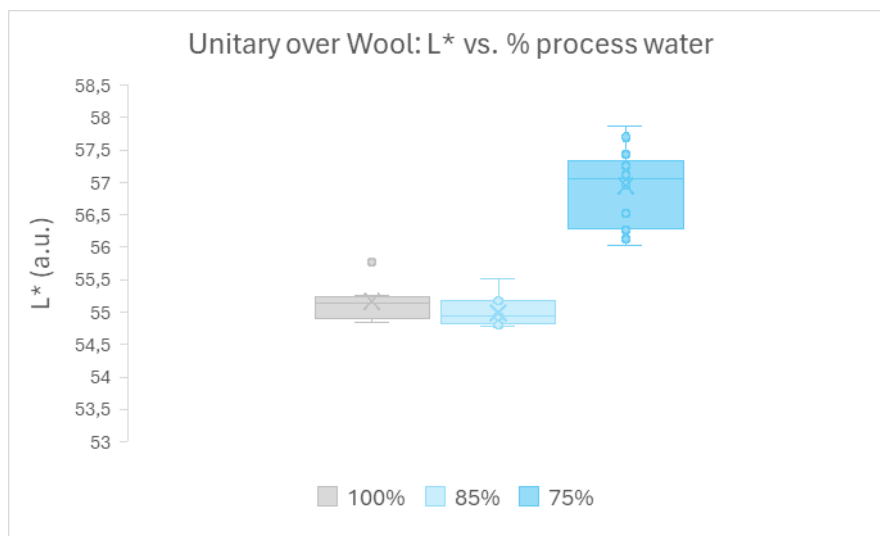


Figure 25. Boxplot for Unitary over Wool fabric: L^* vs. % Process water

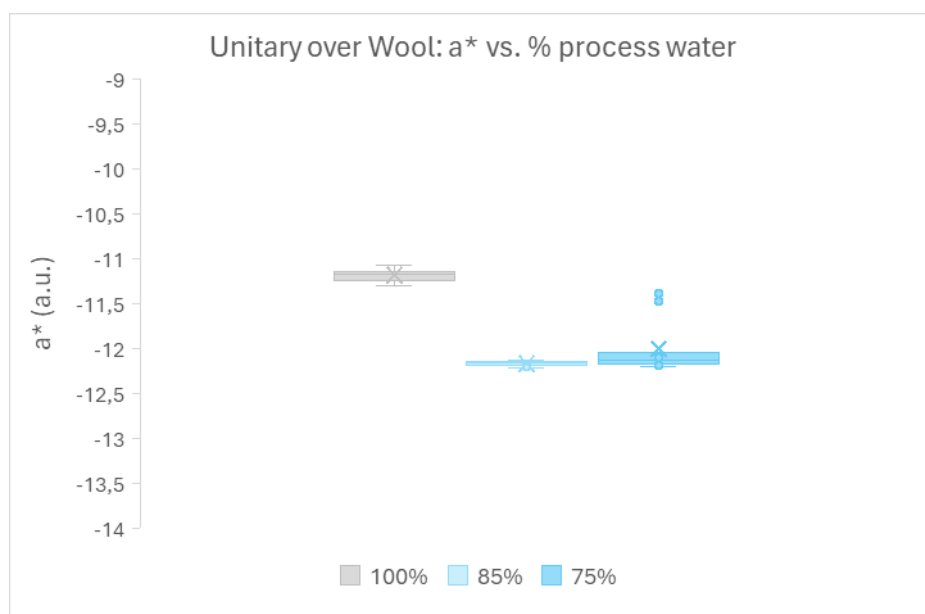


Figure 26. Boxplot for Unitary over Wool fabric: a^* vs. % Process water



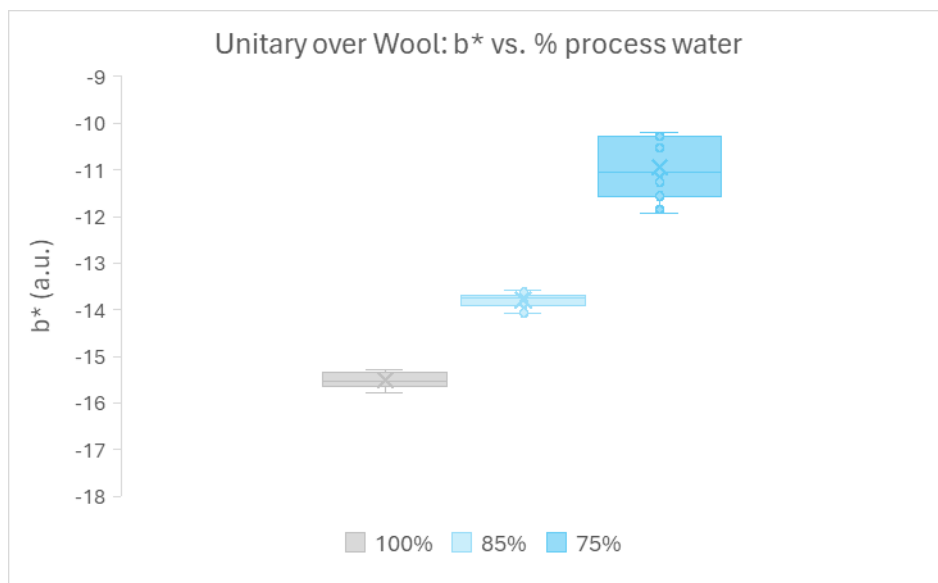


Figure 27. Boxplot for Unitary over Wool fabric: b^* vs. % Process water

Trichromy over Wool fabric

The boxplots below show the L^* , a^* and b^* values obtained from the colourimeter for the 3 experiments: 100% process water (reference), 85% (15% WW from Pilot Plant) and 75% (25% WW from Pilot Plant).

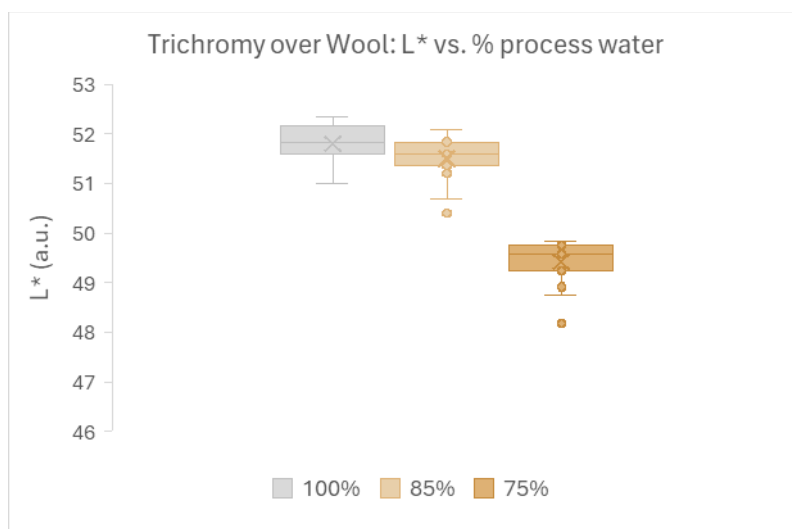


Figure 28. Boxplot for Trichromy over Wool fabric: L^* vs. % Process water



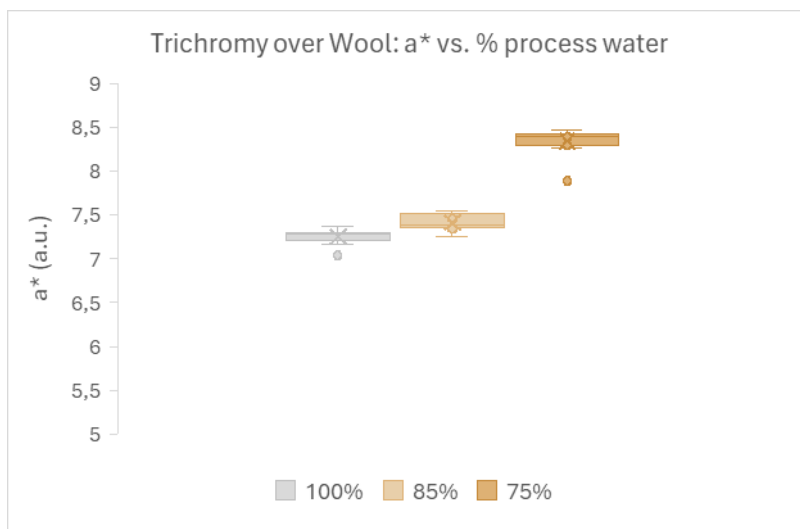


Figure 29. Boxplot for Trichromy over Wool fabric: a^* vs. % Process water

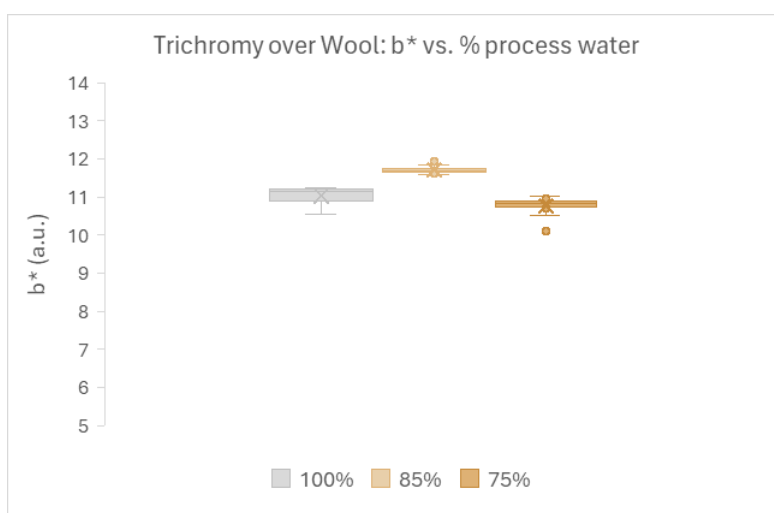


Figure 30. Boxplot for Trichromy over Wool fabric: b^* vs. % Process water

Unitary over polyester yarn

The boxplots below show the L^* , a^* and b^* values obtained from the colourimeter for the 3 experiments: 100% process water (reference), 85% (15% WW from Pilot Plant) and 75% (25% WW from Pilot Plant).



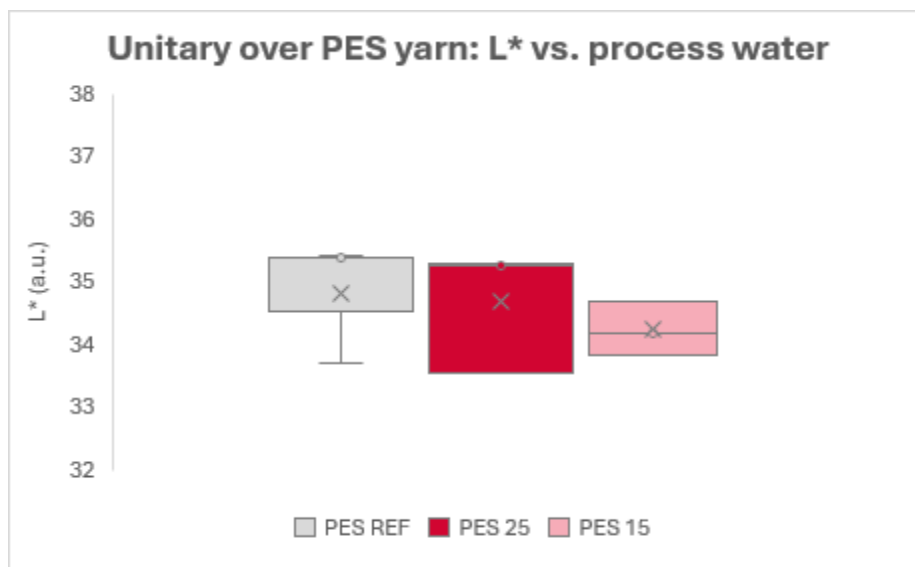


Figure 31. Boxplot for Unitary over Polyester yarn: L* vs. % Process water

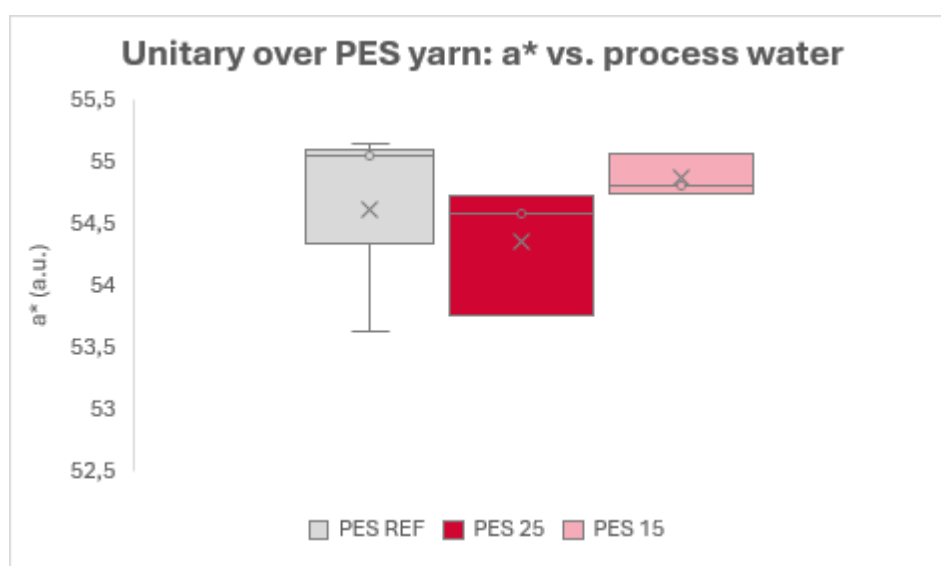


Figure 32. Boxplot for Unitary over Polyester yarn: a* vs. % Process water



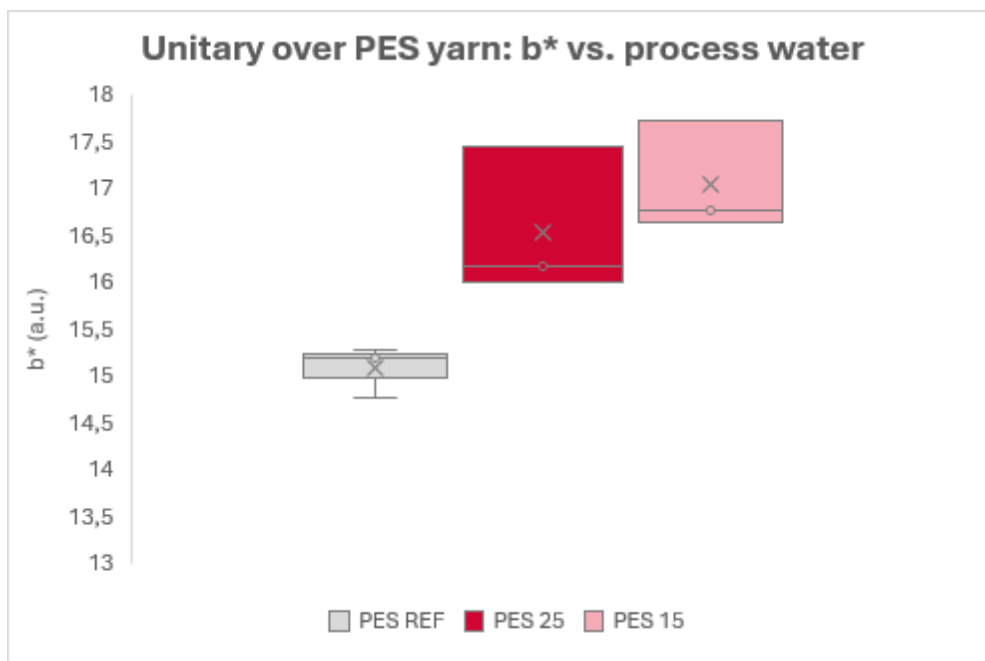


Figure 33. Boxplot for Unitary over Polyester yarn: b* vs. % Process water

Trichromy over polyester yarn

The boxplots below show the L*, a* and b* values obtained from the colourimeter for the 3 experiments: 100% process water (reference), 85% (15% WW from Pilot Plant) and 75% (25% WW from Pilot Plant).

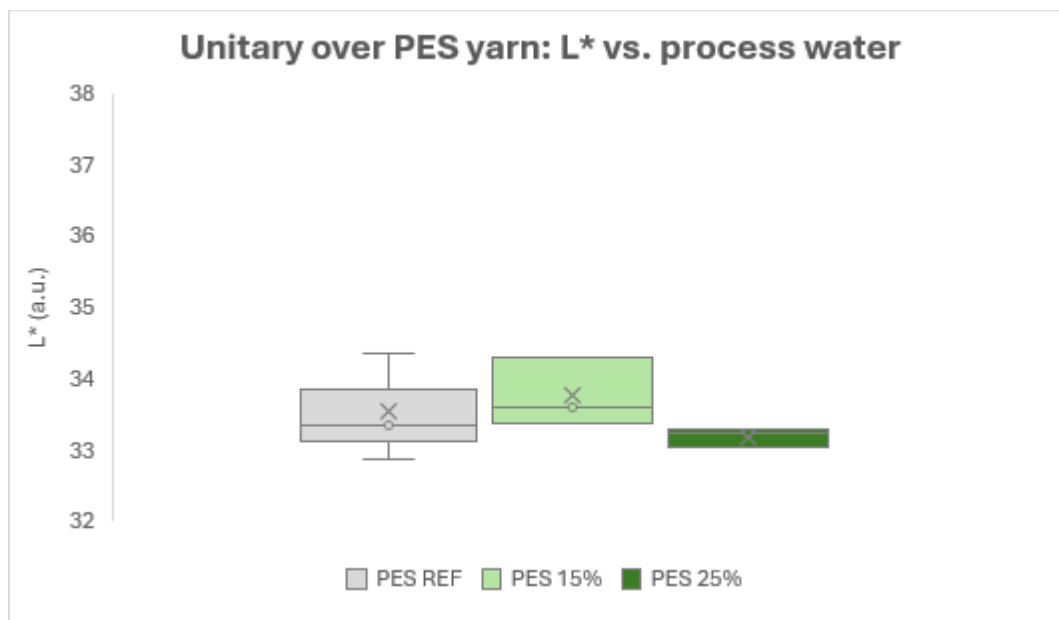


Figure 34. Boxplot for Trichromy over Polyester yarn: L* vs. % Process water



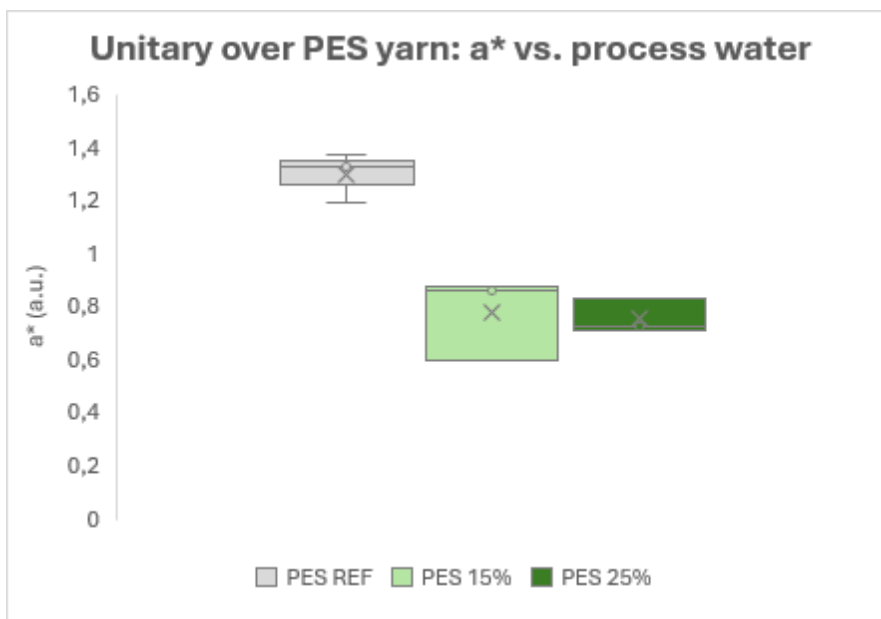


Figure 35. Boxplot for Trichromy over Polyester yarn: a* vs. % Process water

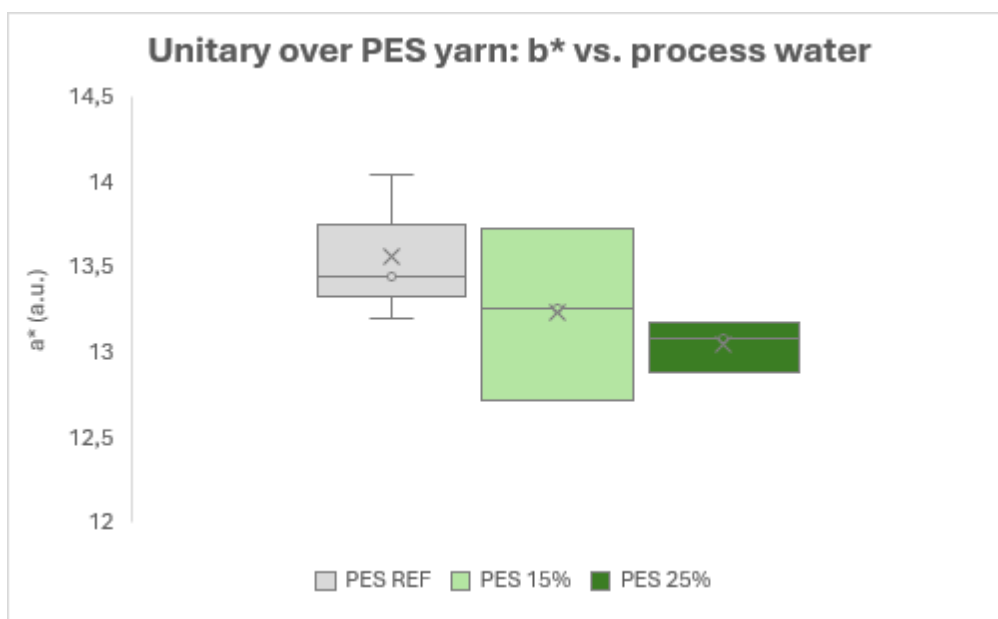


Figure 36. Boxplot for Trichromy over Polyester yarn: b* vs. % Process water

In general, from the graphs above it can be concluded that the values obtained are very similar in the three experiments and the dispersion of the data is not high probing that colour is homogeneous on the surface of the different textile' substrates.





SYMSITES

High Value New Resources validation

Deliverable name: High Value New Resources validation	
ANNEX	D4.4_ANNEX II_GREENE Description of equipment and materials

Content

1.	Description of the work	2
2.	Conclusion	13



1. Description of the work

Background

The recovery of platform molecules from pyrolytic liquids has been part of the SYMSITES strategy for the valorisation of non-recyclable wastes generated in the different EcoSites. Within Task 2.3.2, the project envisaged the upgrading of pyrolysis-derived liquid fractions into valuable compounds such as benzoic acid and acetic acid, through the identification of suitable extraction media, the application of the process to real samples and the investigation of successive separation stages. In this context, the liquid fraction generated during the pyrolysis of selected textile wastes from the Spain EcoSite was identified as a promising source of benzoic acid and related aromatic compounds.

The work previously carried out in D2.5 and completed in D2.6 established the technical basis for this validation activity. First, the pyrolytic liquids were characterised and the aqueous phase obtained after textile pyrolysis was analysed in order to identify the main compounds of interest. On the basis of this characterisation, a liquid-liquid extraction route was selected and optimised for real samples, with dimethyl ether (DME) identified as an appropriate solvent for benzoic acid recovery. The subsequent purification step by recrystallisation confirmed that the recovered solid corresponded to benzoic acid and allowed an improvement in purity suitable for application-oriented work.

The results obtained in WP2 showed that the Spain EcoSite offered the most favourable route for the valorisation of platform molecules. In particular, benzoic acid extraction from the aqueous phase of textile-derived pyrolytic liquid reached an efficiency of 75.2% in two steps, with an estimated material conversion of 81% with respect to the aqueous phase obtained, while the final purification by two-stage recrystallisation yielded a product with 88% purity. On this basis, benzoic acid was selected as the most suitable candidate for further validation in WP4 as a High Value New Resource.

In parallel, the application route associated with this platform molecule was also defined during the previous stages of the project. The selected end-use consisted of transforming the recovered benzoic acid into a formulation suitable for textile finishing, with the aim of preventing fungal growth during storage and contributing to the closing of the loop within the textile value chain. This approach was considered particularly relevant for the Spain EcoSite, since it linked a molecule recovered from textile waste pyrolysis with a new functional use in textile materials.

Validation approach

The starting point of the validation approach was the characterisation of the liquid fraction generated after pyrolysis of the selected textile waste stream. Earlier work in SYMSITES showed that the pyrolytic liquid separated into an aqueous phase and an organic phase, with the aqueous phase concentrating several oxygenated and aromatic compounds of potential interest. This phase was considered particularly relevant because, despite its complex composition, it contained compounds that could be recovered through low-temperature downstream separation routes, thereby improving the overall valorisation of the pyrolysis process.

For the Spain EcoSite, the aqueous phase obtained from textile scraps was analysed by GC-MS in order to identify the dominant compounds and to evaluate whether a realistic platform molecule route could be built around them. The chromatographic profile showed that benzoic acid and benzoic derivatives were the main species of interest in the textile-derived liquid stream. This was a decisive result, because benzoic acid combines three characteristics that make it especially suitable for validation in the framework of SYMSITES: it can be recovered from the real pyrolytic liquid, it can be purified by conventional laboratory operations, and it has a direct functional application in sectors connected to the textile value chain.



The choice of benzoic acid was therefore based on both analytical evidence and practical relevance. Unlike more diffuse families of compounds that would have required broad fractionation or market reclassification, benzoic acid offered a focused and technically traceable route from waste to product. Its selection also matched the logic of WP4, which aimed to validate High Value New Resources in relevant operational conditions rather than merely reporting laboratory observations. In other words, the molecule was selected because it could realistically be taken one step further and assessed as a candidate functional raw material.



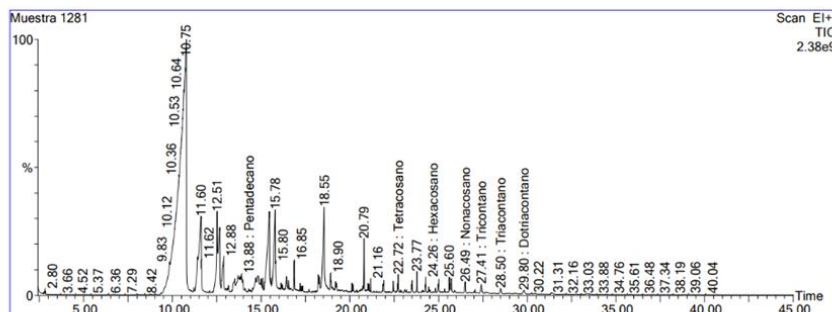
Figure 1. Different fractions obtained after pyrolysis of textile and biomass-derived non-recyclable waste, used as the starting streams for platform molecule recovery.

Once benzoic acid had been identified as the target platform molecule, the next step consisted of selecting and validating a separation route suitable for the real aqueous phase generated after pyrolysis. Given the composition of the stream and the need to avoid unnecessarily harsh or complex operations, the approach selected in SYMSITES was liquid-liquid extraction. This method was considered appropriate because it offered a direct way to transfer benzoic acid from the aqueous phase to an organic solvent phase, while remaining simple enough to be developed at laboratory scale and conceptually transferable to a larger-scale operation.



Qualitative Report

File: C:\TurboMass\Instalacion.PRO\Data\Muestra 1281.raw
 Acquired: 13-Dec-23 01:00:11 PM
 Description: Con acetato de etilo
 GC/MS Method: GC: Aceites 45min_Manual.mth MS: Aceites 45min.EXP
 Sample ID: D26-COND-793-S0052
 Printed: 13-Dec-23 01:46 PM
 Page 1 of 1
 Vial Number: 10



#	RT	Height	Area	Area %	Norm %	Nombre cuant	Nombre NIST
1	10.783	2,315,499,008	960,912,064	74.0	100.00		Benzoic acid
2	11.604	687,031,360	112,162,128	8.6	11.67		Benzoic acid, 4-methyl-
3	15.455	669,725,312	85,220,440	6.6	8.87		Benzoic acid, 2-(1-oxopropyl)-
4	15.781	721,656,448	71,643,544	5.5	7.46		Isophthalic acid
5	18.547	743,965,688	68,910,136	5.3	7.17		Biphenyl-4-carboxylic acid
		5137878016	1298848312	100.01			

Figure 2. GC-MS results for the aqueous phase obtained from textile waste pyrolysis in the Spain EcoSite, showing benzoic acid and benzoic derivatives as the main compounds of interest.

Among the solvents examined in the previous stages of the project, dimethyl ether (DME) was selected as the most appropriate option for benzoic acid recovery from the textile-derived aqueous phase. The choice was driven by the observed extraction behaviour, the capacity to work at low temperature, and the practical advantage that DME can be readily separated and recovered after extraction. From a process integration perspective, this was important because the SYMSITES logic was not only to obtain a solid product but also to do so through a route that could later be framed as cost-efficient and circular.

The recovery route was therefore structured as a DME-based liquid-liquid extraction, followed by solvent removal and recovery of the extracted solid. This sequence allowed the project to move from an analytically identified target compound to a physically isolated product. It also offered a robust basis for reproducibility checks and for the subsequent purification stage.

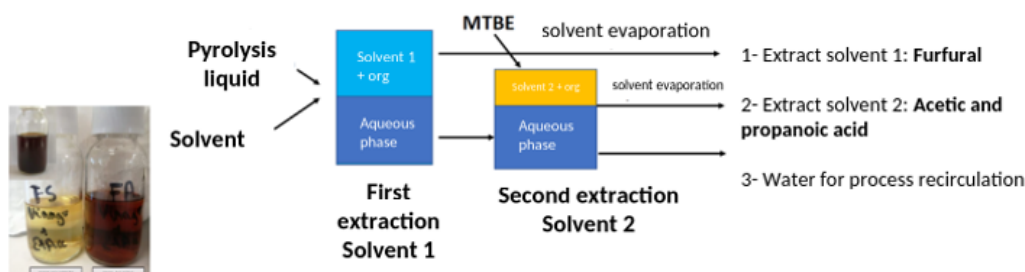


Figure 3. Liquid-liquid extraction selected as the most suitable route for benzoic acid recovery from the textile-derived aqueous phase.

The recovery tests confirmed that DME is able to extract benzoic acid efficiently from the real aqueous phase produced by textile waste pyrolysis. In one extraction step, an average extraction efficiency of around 50% was obtained. When the extraction was repeated in a second stage with fresh solvent, the cumulative extraction efficiency increased to 75.2%. This result was particularly relevant because it showed that a relatively simple cascade strategy could significantly improve product recovery without changing the core process. The estimated material conversion reached 81% with respect to the total aqueous phase obtained, which reinforced the idea that the textile-derived liquid stream can indeed function as a realistic source of a recoverable platform molecule.

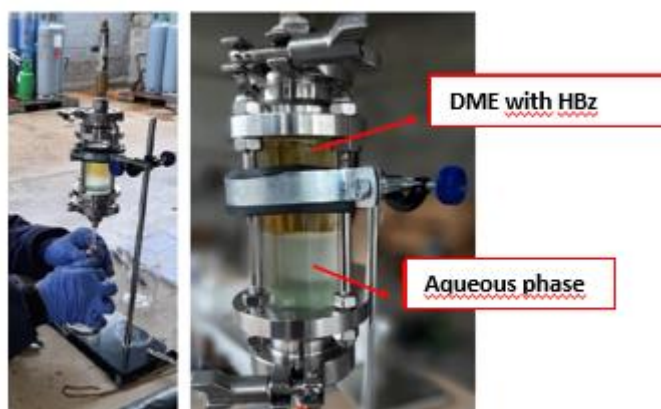


Figure 4. Extraction phase scheme illustrating the transfer of benzoic acid from the aqueous phase to the DME-rich phase.

The recovery tests confirmed that DME is able to extract benzoic acid efficiently from the real aqueous phase produced by textile waste pyrolysis. In one extraction step, an average extraction efficiency of around 50% was obtained. When the extraction was repeated in a second stage with fresh solvent, the cumulative extraction efficiency increased to 75.2%. This result was particularly relevant because it showed that a relatively simple cascade strategy could significantly improve product recovery without changing the core process. The estimated material conversion reached 81% with respect to the total aqueous phase obtained, which reinforced the idea that the textile-derived liquid stream can indeed function as a realistic source of a recoverable platform molecule.

Recovering a solid after solvent evaporation was a necessary milestone, but it was not sufficient by itself for validation under application-oriented conditions. The project also needed to determine whether the recovered solid could be upgraded to a quality level suitable for formulation work. For this reason, the extraction stage was followed by a purification step based on recrystallisation.

Recrystallisation was selected because it is a standard and reliable method for improving the purity of organic solids when the main compound is already present at high proportion. In the SYMSITES case, the extracted benzoic acid showed a sufficiently high initial content to make such purification worthwhile. The operation therefore aimed to reduce the presence of co-extracted impurities and to generate a product whose identity and quality could be more confidently associated with benzoic acid.





Figure 5. Experimental process followed for benzoic acid recrystallisation.

The purification sequence showed a clear improvement in product quality. The initial solid obtained after extraction presented a purity around 74%, and after two recrystallisation stages the final purity reached 88%. This result was highly relevant for the validation pathway because it demonstrated that the recovered platform molecule was not only technically extractable but also technically upgradable through a relatively simple downstream operation. In practical terms, the recrystallisation step provided the bridge between recovery and functional validation.

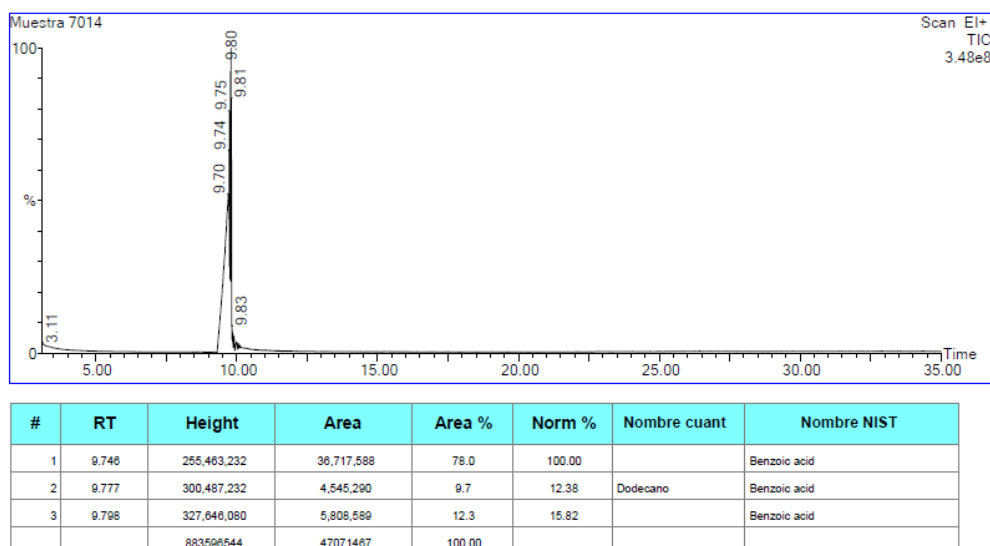


Figure 6. GC-MS profile of the recrystallised benzoic acid, confirming the improvement in product quality after purification.

Once the molecule had been recovered and purified, the validation approach moved from chemical processing to functional application. For the Spain EcoSite, the selected end-use scenario was the preparation of a formulation capable of providing anti-odour and antifungal protection to textile



materials during storage. This application was considered particularly suitable because the molecule recovered from textile waste pyrolysis could be reintroduced into the textile value chain as a functional additive, thereby reinforcing the industrial-urban symbiosis logic of the project.

The first formulation concept was based on converting benzoic acid into sodium benzoate through neutralisation with sodium hydroxide. The rationale for this choice was practical: sodium benzoate is easier to handle in aqueous systems and is widely known as a preservative. However, this route soon revealed an important formulation challenge. Although sodium benzoate is industrially familiar, the biologically active species responsible for antimicrobial behaviour is associated mainly with the acidic form of the molecule. Consequently, formulation pH became a critical variable in the validation strategy.

The initial tests were therefore not only designed to assess performance, but also to identify the chemical conditions under which the recovered benzoic acid could express meaningful antifungal activity. In that sense, the validation approach was deliberately iterative: it was expected that the first trials might reveal limitations, and those limitations would then guide a more appropriate formulation strategy.

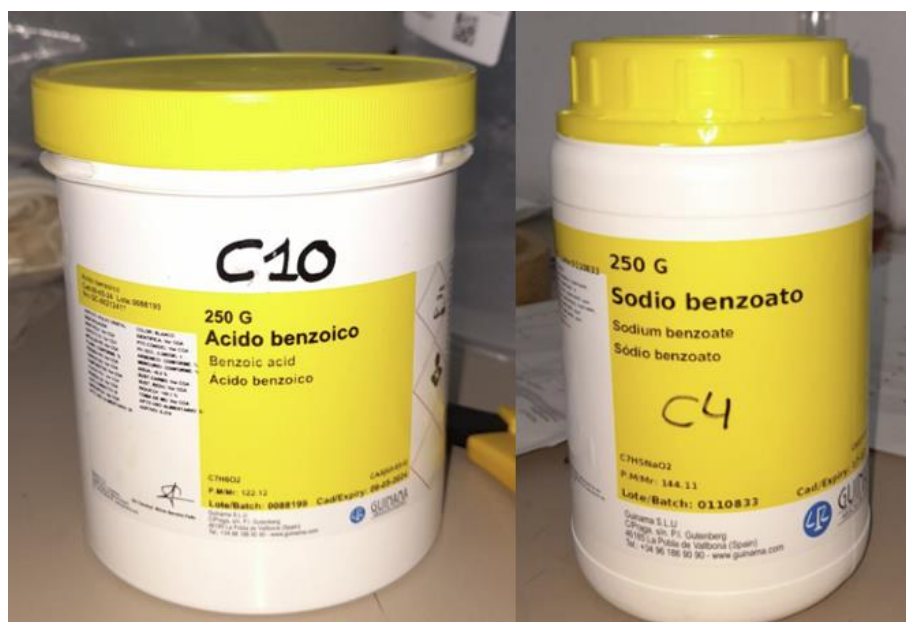


Figure 7. different acids used as starting material for the preparation of the validation formulation.

In the first validation stage, sodium benzoate solutions were prepared in water at pH 5.5 and applied to cotton knitted fabric by foulard impregnation. The process conditions included a final solution volume of 400 mL, a benzoate concentration of 1.25 g/L, a drying temperature of 100 °C, a drying time of 4 minutes, an estimated pick-up of 80% and an application pressure of 40 psi. The treated fabric was then analysed for antifungal performance against *Candida albicans* and *Aspergillus niger*.

This first stage played an important role in the overall validation approach because it established the baseline behaviour of the formulation under relatively mild and industrially realistic application conditions. It also enabled the direct comparison between untreated textiles, textile treated with sodium benzoate reference material, and textile treated with benzoate derived from benzoic acid.

The outcome of this first stage was negative from a functional point of view. The formulation did not prevent fungal growth under the conditions tested. This result, however, was not a failure of the

validation approach; on the contrary, it provided critical information. It showed that sodium benzoate at pH 5.5 is not a sufficient route to transfer antifungal activity to the textile substrate. This immediately redirected the work towards understanding the role of pH and the acid-base equilibrium of benzoic acid.

Fotografía con el microscopio estereoscópico
Photography with the stereoscopic microscope

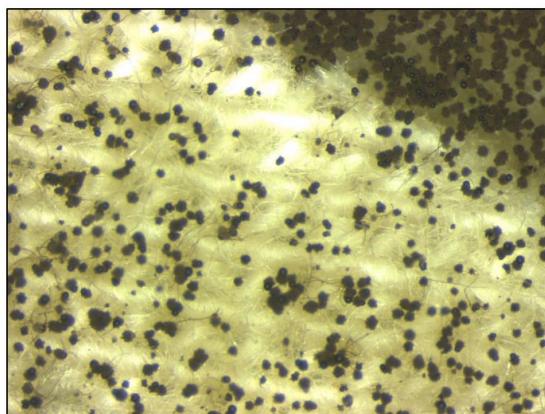


Figure 8. Foulard equipment used for textile impregnation during the first validation stage.

Crecimiento sobre la muestra Growth upon the sample	
Valoración Assesment	Rango de crecimiento ⁽¹⁾ Growth ratio ⁽¹⁾
Hay crecimiento y es visible para el ojo humano (crecimiento macroscópico) There is growth and visible to the human eye (macroscopic growth)	5

Fotografía de la muestra ensayada
Photograph of the tested sample



Figure 9. Cotton fabric after foulard application and drying of the benzoate-based formulation.

Valoración Assesment	Rango de crecimiento Growth ratio
No hay crecimiento No growth	0
Hay crecimiento, pero solo visible al microscopio (crecimiento microscópico) There is growth, but only visible to the microscope (microscopic growth)	1
Hay crecimiento y es visible para el ojo humano (crecimiento macroscópico) There is growth and visible to the human eye (macroscopic growth)	Trazas (menos del 10%) 2
	Crecimiento bajo (10-30%) 3
	Light growth (10 to 30%) 3
	Crecimiento medio (30-60%) 4
	Medium growth (30 to 60%) 4
	Crecimiento alto (> 60%) 5
	Heavy growth (> 60%) 5

Figure 10. Growth assessment scale used for the interpretation of the antifungal results.

Following the poor performance of the first formulation, additional analysis was carried out to understand why the benzoate-based system did not exhibit sufficient antifungal behaviour. The key observation was that antimicrobial activity is linked primarily to the acidic form of benzoic acid. At pH 5.5, only a very small fraction of the molecules remains in the non-dissociated acidic form, which explains why the first tests did not produce the expected result.

To address this issue, the formulation strategy was redesigned. The target was to reduce the pH to around 4.7 and to use a mixed solvent system combining water and propylene glycol. The presence of propylene glycol was intended to improve the handling and apparent stability of the acidic form, while citric acid was used to adjust the final pH. The resulting solution, referred to as AF_1, contained 44% water, 44% propylene glycol, 4.8% sodium benzoate and 7.2% citric acid solution in its initial laboratory version.

Before reapplying the formulation to textiles, the project first assessed its antifungal behaviour in solution according to AATCC Test Method 30-2017 against *Candida albicans* and *Aspergillus niger*. This intermediate step was essential from a validation perspective. It allowed the intrinsic biological response of the formulation to be evaluated separately from the textile application process, thereby distinguishing between formulation limitations and deposition limitations.

Under these acidic conditions, the reformulated AF_1 solution showed no fungal growth for either *Candida albicans* or *Aspergillus niger*. This was a decisive result, because it confirmed that the recovered benzoic acid could indeed generate an antifungal formulation when the pH and solvent environment were properly adjusted. The formulation challenge was therefore not whether the molecule had antifungal potential, but how to retain or transfer that potential onto the textile surface after application and drying.

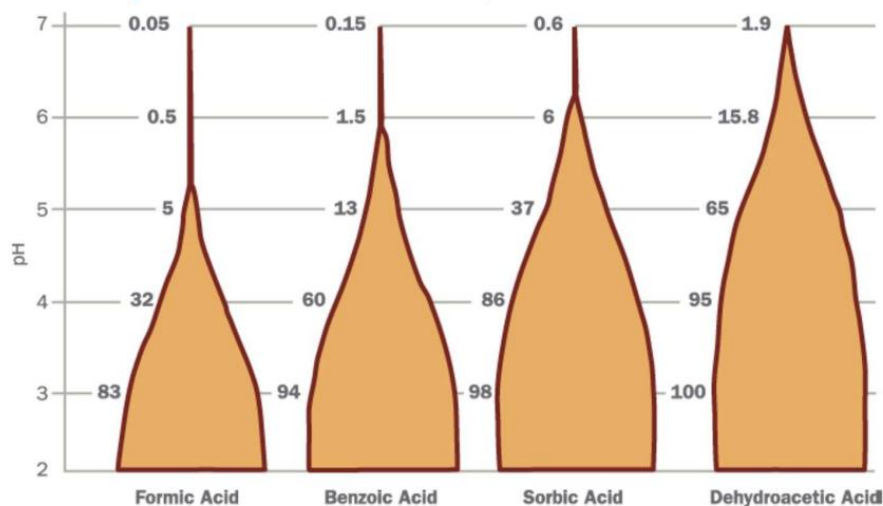


Figure 11. Dissociation behaviour of benzoic acid as a function of pH, supporting the need for acidic conditions to favour the active form.

After confirming antifungal activity in solution, the AF_1 formulation was applied again to textile substrates using foulard impregnation. In this second round of textile validation, the sodium benzoate concentration increased to 48 g/L, with the pH kept close to 4.5, and the same drying conditions were maintained. The first application was carried out at 40 psi.

The results obtained after this impregnation showed that the antifungal response observed in solution was not fully preserved on the fabric. Growth was still detected after incubation. This



suggested that either the number of active species retained on the fabric was insufficient or that the drying and distribution conditions reduced the effective concentration available at the textile surface.

To improve deposition, the foulard application was repeated at a lower pressure of 30 psi, with the intention of increasing the pick-up. This modification led to better results, particularly against *Aspergillus niger*, for which the growth ratio decreased to a trace level rather than the stronger growth observed in the previous test. Nevertheless, fungal growth was still not completely suppressed. These observations confirmed that the application route was highly sensitive to process conditions and that further optimisation was required.



Figure 12. Example of treated textile appearance after application of the benzoate/benzoic acid-based formulation.

Because the foulard route did not ensure sufficient transfer of antifungal performance to the textile substrate, the validation strategy incorporated a third application route based on spraying. The reason for this change was straightforward: spraying offered the possibility of depositing a higher amount of solution directly onto the textile surface, with less squeezing-out than in foulard impregnation. In the trials performed, the AF_1 solution was sprayed onto both polyester and cotton fabrics and left to air dry.

The resulting pick-up values were significantly higher than in the foulard route, reaching approximately 126% for polyester and 134% for cotton. These conditions were then assessed microbiologically according to AATCC Test Method 30-2017 (Method III). The spraying route generated the clearest positive outcome of the entire application stage: no growth of *Candida albicans* was observed on either polyester or cotton fabric. This showed that, when enough formulation was deposited on the surface, the benzoic acid-based system was capable of producing a meaningful antifungal response against at least one of the two target microorganisms.

However, the same route was not effective against *Aspergillus niger*. In that case, both polyester and cotton still showed a high growth ratio, corresponding to visible macroscopic growth. This contrast between the two microorganisms is a highly informative result. It indicates that the recovered and formulated benzoic acid has real antifungal functionality, but that this functionality is selective and strongly dependent on the target species, the deposited amount and the conditions of application.

From a validation perspective, the spraying route therefore provided a partial but important success: it demonstrated the practical potential of the recovered platform molecule on textile substrates, while



at the same time revealing the limits that still need to be addressed before a fully robust textile preservation treatment can be claimed.

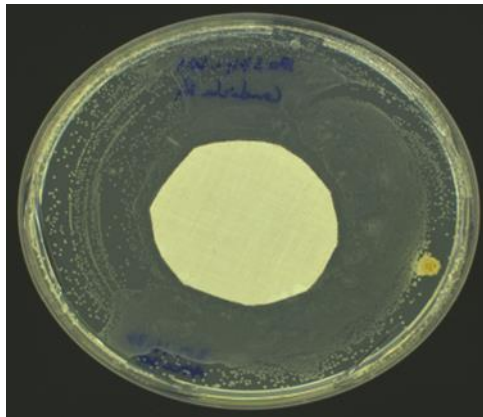


Figure 13. Polyester sample after spray application and microbiological testing against *Candida albicans*.

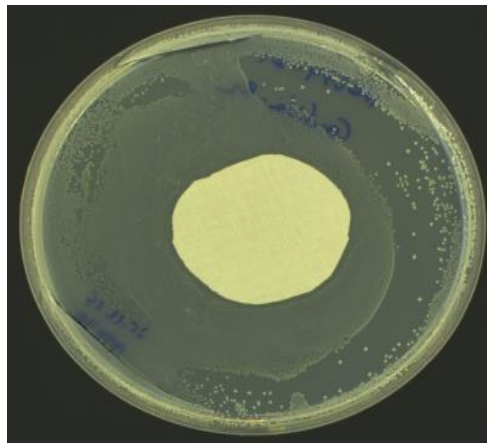


Figure 14. Cotton sample after spray application and microbiological testing against *Candida albicans*.

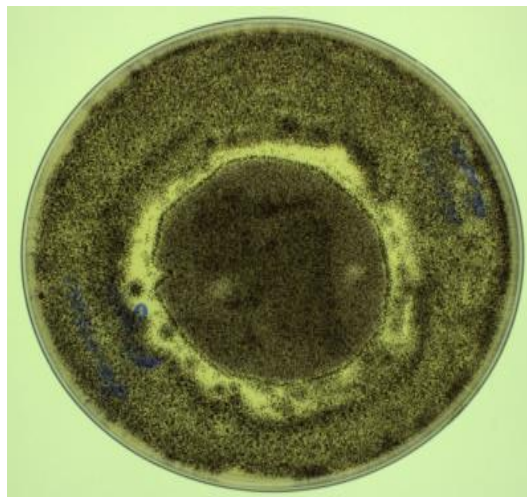


Figure 15. Polyester sample after spray application and microbiological testing against *Aspergillus niger*.

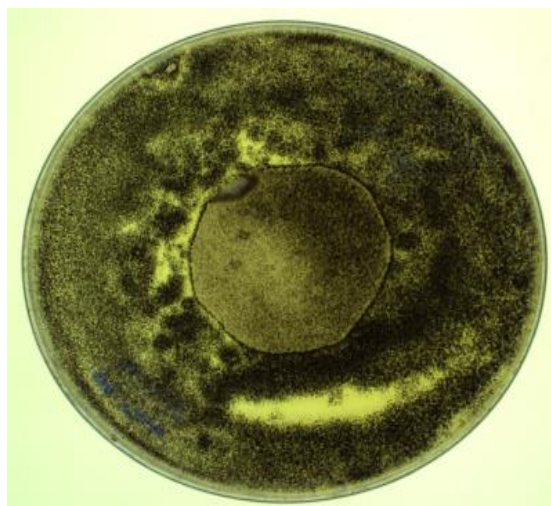


Figure 16. Cotton sample after spray application and microbiological testing against *Aspergillus niger*.

Taken as a whole, the validation approach followed for benzoic acid in SYMSITES can be regarded as a structured progression from recovery to application. The work first demonstrated that textile-derived pyrolytic aqueous phase is a credible source of benzoic acid, then showed that the molecule can be extracted with DME, purified by recrystallisation and converted into a formulation with confirmed antifungal activity in solution. Only after those stages were successfully completed did the project move towards textile application trials.

This sequential pathway is one of the main strengths of the work. It means that the limitations identified at the final stage cannot be attributed to a lack of molecular recovery or to a lack of intrinsic functionality of the acidic benzoic system. Instead, the remaining challenge lies primarily in the transfer of that functionality to the textile substrate in a sufficiently stable and effective manner. The work therefore narrows down the optimisation needs and provides a solid technical basis for future improvement.

A second relevant outcome is that the validation route clearly discriminated between formulation conditions. The difference between the first sodium benzoate solution at pH 5.5 and the acidic AF_1 formulation at pH around 4.7 was critical. This proves that chemical speciation must be considered as a central design variable if pyrolysis-derived benzoic acid is to be used as a functional ingredient.

Finally, the results highlight that the most credible claim emerging from this section is not that a fully commercial textile preservative has already been achieved, but rather that SYMSITES has demonstrated a realistic and traceable route for converting a secondary pyrolysis stream into a purified platform molecule with measurable functional performance. That is fully aligned with the concept of High Value New Resources within the project.

The validation approach for platform molecules, centred on benzoic acid from textile-derived pyrolytic aqueous phase, has shown that the route developed by GREENE is technically coherent from start to finish. The molecule was selected on the basis of real chromatographic evidence, recovered through a suitable extraction route, purified to a significantly improved quality level and reformulated for functional assessment.

The antifungal validation work demonstrated that the benzoic acid/benzoate system has clear activity in solution under acidic conditions and that this activity can be transferred, at least partially, to textile substrates. The strongest positive result was obtained against *Candida albicans* after spray

application on polyester and cotton. By contrast, performance against *Aspergillus niger* remained insufficient under the tested textile application conditions. This means that the route is promising and validated at proof-of-concept level but still requires optimisation of deposition strategy and formulation robustness for broader antifungal protection.

2. Conclusion

The work carried out in this part of D4.4 has validated the platform molecule route developed in SYMSITES for the Spain EcoSite, confirming that the aqueous phase obtained from the pyrolysis of textile-derived non-recyclable waste can be upgraded into a high value resource with potential functional application. In particular, the previous work developed in WP2 demonstrated that benzoic acid is the main target compound present in the aqueous phase of the textile-derived pyrolytic liquid and that it can be effectively recovered by liquid-liquid extraction using dimethyl ether (DME). The extraction route provided a recovery efficiency of 75.2% in two steps, with an estimated material conversion of 81% with respect to the aqueous phase obtained, and the subsequent purification by recrystallisation yielded a final product with a purity of 88%. These results confirmed the technical feasibility of the recovery and purification route and provided a sufficiently robust basis for the application-oriented validation carried out in WP4.

The validation activities also confirmed that the recovered benzoic acid can be transformed into a formulation of interest for the textile sector, supporting its consideration as a High Value New Resource within the SYMSITES Industrial-Urban Symbiosis concept. The selected validation route, focused on the use of benzoic acid for the protection of textiles during storage, was technically relevant because it enabled the reintroduction of a molecule recovered from textile waste pyrolysis into the textile value chain through a new functional use. In this respect, the work demonstrated that the final functionality of the recovered molecule depends not only on its extraction and purity, but also on the chemical form of the active compound, the pH of the formulation, and the application method used on the textile substrate.

The first validation tests showed that sodium benzoate formulated in water at pH 5.5 did not provide antifungal performance under the selected conditions, indicating that the benzoate salt was not sufficiently active in this medium. This first result was important because it allowed the validation strategy to be refined on a sound technical basis. Further analysis showed that the antimicrobial activity is associated mainly with the acidic form of benzoic acid, which led to the development of a second formulation based on a water/propylene glycol mixture adjusted to acidic pH with citric acid. Under these revised conditions, the liquid formulation showed positive antifungal activity against both *Candida albicans* and *Aspergillus niger*, confirming that the recovered benzoic acid can generate an active preservative system when the formulation conditions are properly adjusted.

When the formulation was transferred from liquid screening to textile application, the results were more uneven. The tests performed by foulard impregnation showed that the antifungal effect on the treated fabric was limited, although a clear improvement was observed when the application conditions were modified in order to increase the product uptake. The final trials carried out by spraying provided the most promising textile results, showing no growth of *Candida albicans* on both polyester and cotton fabrics. However, the same treatment remained insufficient to prevent the growth of *Aspergillus niger*, for which a high growth ratio was still observed on both substrates. Therefore, the validation work demonstrated a partial but relevant proof of concept: the recovered



benzoic acid has clear antifungal potential, but its final effectiveness on textile substrates is microorganism-dependent and still conditioned by the efficiency of deposition and retention on fabric.

The work reported here confirms that the platform molecules route in SYMSITES is technically credible and that benzoic acid recovered from textile-derived pyrolytic liquids is a valid candidate for further valorisation as a circular product. The results support the concept that pyrolysis liquid streams can generate not only intermediate fractions, but also functional molecules with practical industrial interest. At the same time, the validation has identified the main technical limitations that must be addressed to consolidate this route, namely the optimisation of formulation chemistry and, especially, the improvement of textile application procedures in order to enhance antifungal performance on the final material. In this sense, the work carried out in D4.4 constitutes a solid validation step from molecule recovery to application-oriented assessment, while also defining the areas where further optimisation would be required before industrial implementation.





SYMSITES

High Value New Resources validation

Deliverable name: High Value New Resources validation	
ANNEX	D4.4_ANNEX III_NTUA Description of equipment and materials

Content

1.	Hydrogen production.....	2
1.1	Analytical methods	2
1.2	Quantification of parameters small scale reactor	4



1. Hydrogen production

1.1 Analytical methods

Determination of hydrogen composition

Hydrogen productivity was calculated as the product of the volumetric flow rate of the produced biogas and the hydrogen fraction in the gaseous mixture. The latter was determined through gas sampling at the top of the bioreactor. Gas samples were collected throughout the experiments using appropriate glass sampling devices equipped with a septum, transferred to the laboratory, and analyzed using an SRI 8610C Gas Chromatograph equipped with a thermal conductivity detector (TCD) and packed columns. Specifically, the chromatograph (MG#1) was fitted with two columns connected in series: a molecular sieve column (6 ft, O.D. 1/8 in., I.D. 2.1 mm) and a silica gel column (6 ft, O.D. 1/8 in.). For hydrogen determination, nitrogen (N₂) was used as the carrier gas at a flow rate of 20 mL min⁻¹. The temperatures of the column oven, injection valve, and detector were maintained at 80 °C, 90 °C, and 100 °C, respectively.

Gas composition was determined using calibration curves constructed from standard gas mixtures with hydrogen concentrations of 10%, 20%, 40%, 60%, 80%, and 100%. Based on these measurements, the calibration curve described by Equation (1) was obtained.

$$\text{H}_2 (\%) = 0.0151 \times \text{area counts} - 1.5417 \text{ (Eq. 1)}$$



Figure 1. The Gas Chromatography used for the hydrogen composition measurement

Determination of volatile fatty acids (VFAs) – Ethanol

The determination of VFAs and ethanol was carried out using a Shimadzu Nexis GC-2030 Gas Chromatography system equipped with a flame ionization detector (FID) and a capillary column HP-FFAP (Agilent Technologies Inc., 30 m × 0.53 mm I.D. × 1.00 µm film). The samples to be analyzed are first filtered, and then 1 mL of the sample is acidified with 30 µL of 20% v/v H₂SO₄. Subsequently, 10 µL of the acidified solution is injected into the chromatograph using an autosampler microsyringe at a temperature of 175 °C. The temperature program for VFA separation starts at 105 °C, increasing at a rate of 15 °C min⁻¹ up to 160 °C. Then, the rate increases to 20 °C min⁻¹ up to 235 °C, where the temperature is held constant for 3 minutes. The detector temperature was set at 225 °C.

For ethanol measurement, the injection temperature is initially held at 60 °C for 1 minute and then increased at a rate of 45 °C min⁻¹ up to 230 °C, where it is maintained for 0.5 minutes. The final detector temperature is set at 200 °C.





Figure 2. The Gas Chromatography used for the volatile fatty acids (VFAs) measurement

Determinations of total and soluble chemical oxygen demand (TCOD and sCOD)

COD is defined as the equivalent amount of oxygen required to oxidize the constituents of a sample using a strong oxidizing agent. COD determination is carried out using the closed reflux method, as described in Method 5220D of Standard Methods for the Examination of Water and Wastewater. In this method, the Cr^{3+} ions produced from the oxidation of organic matter are measured photometrically at 600 nm. Thus, an excess of potassium dichromate ($\text{K}_2\text{Cr}_2\text{O}_7$) oxidizes the organic sample under strongly acidic conditions, with heating at 148 °C. Silver sulfate (Ag_2SO_4) is used as a catalyst for the oxidation of aliphatic compounds, while mercury sulfate (HgSO_4) is added to prevent the binding of silver ions with halogenated substances present in the waste, which would otherwise form precipitates upon complexation.

The concentrated sulfuric acid solution is prepared by dissolving 25.3 g of Ag_2SO_4 in 2.5 L of H_2SO_4 . The potassium dichromate reagent is prepared by dissolving 10.216 g of $\text{K}_2\text{Cr}_2\text{O}_7$ (dried at 103 °C for 2 h), 167 mL of concentrated H_2SO_4 , and 33.3 g of HgSO_4 in 500 mL of deionized water. After thorough mixing, the solution is diluted to 1 L and stored in a dark bottle at room temperature.

During the analysis, 2 mL of the appropriately diluted sample (with deionized water) is placed in special vials. Then, 1.2 mL of potassium dichromate solution and 2.8 mL of catalyst solution are added. Digestion is performed for two hours at 148 °C in a thermostatically controlled reactor (Hach DRB 200). After cooling, the vials are measured photometrically using a spectrophotometer (Hach DR 5000).

Quantification of COD concentration is achieved using a calibration curve constructed from absorbance data versus concentrations of standard potassium hydrogen phthalate (KHP) solutions. Initially, a standard KHP solution of 0.850 g L^{-1} (previously dried at 120 °C) is prepared, corresponding to $1000 \text{ mg O}_2 \text{ L}^{-1}$. From this, standard solutions with concentrations of 20, 50, 100, 250, 800, and $1000 \text{ mg O}_2 \text{ L}^{-1}$ are prepared.

Determination of Carbohydrates

The method of Josefsson (1983) was used for the determination of carbohydrates, which react with L-tryptophan in the presence of sulfuric and boric acid. Complex formation occurs between the polyaromatic compounds of the reagent and the sugar molecules of the sample, resulting in a brown-violet color for hexoses or a brown-green color for pentoses. With this method, it is also possible to



determine complex carbohydrates (e.g., cellulose), as concentrated sulfuric acid has the ability to hydrolyze glycosidic bonds of polymers.

For the analysis, 4 mL of reagent is added to 2 mL of the sample, which has been appropriately diluted with deionized water to ensure that its concentration falls within the method's range. The vial is sealed, mixed using a vortex, and digested in a water bath for 20 minutes. After cooling, a photometric measurement is carried out at 520 nm.

The carbohydrate content in each sample is calculated using a calibration curve similar to that used for COD determination; however, the working range is 5–100 mg L⁻¹.

Determination of Solids

Total suspended solids (TSS) and volatile suspended solids (VSS) measurements were carried out according to Methods 2540D and 2540E, respectively, of the Standard Methods for the Examination of Water and Wastewater. TSS is defined as the amount of non-filterable solids retained on a pre-weighed glass fiber filter. The filtered sample volume is known and properly homogenized. The filter is then dried to constant weight at 103–105 °C, and the increase in weight corresponds to the TSS. VSS represents the amount remaining after ignition of the TSS at 550 °C until constant weight is achieved. The reduction in the filter weight corresponds to the VSS.

Determination of pH

The measurement of the pH was done using a HANNA (pH 211) pH-meter with a HANNA electrode (HI 1230).

1.2 Quantification of parameters small scale reactor

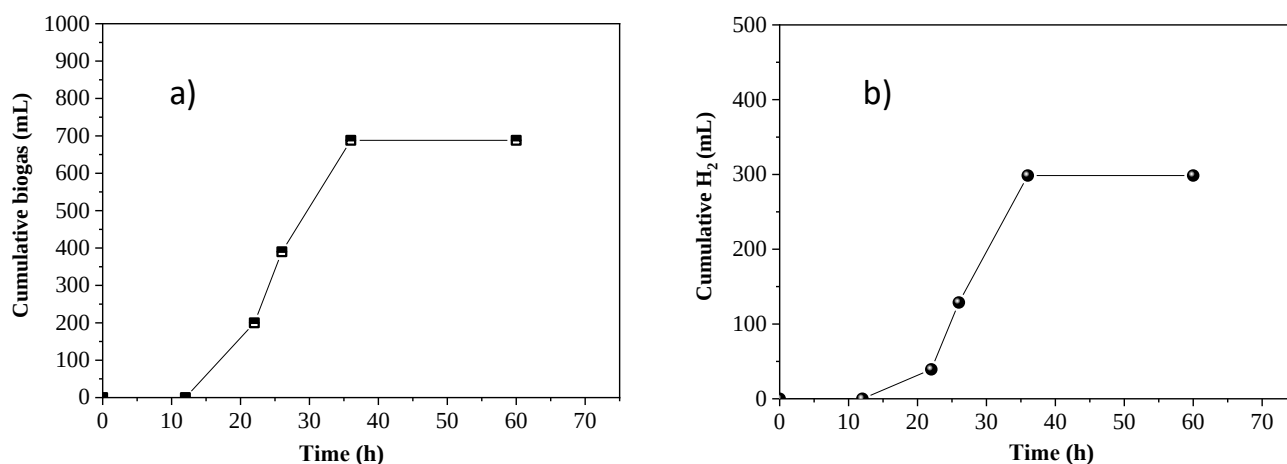


Figure 3. Cumulative biogas (a) and hydrogen production (b) in batch1.

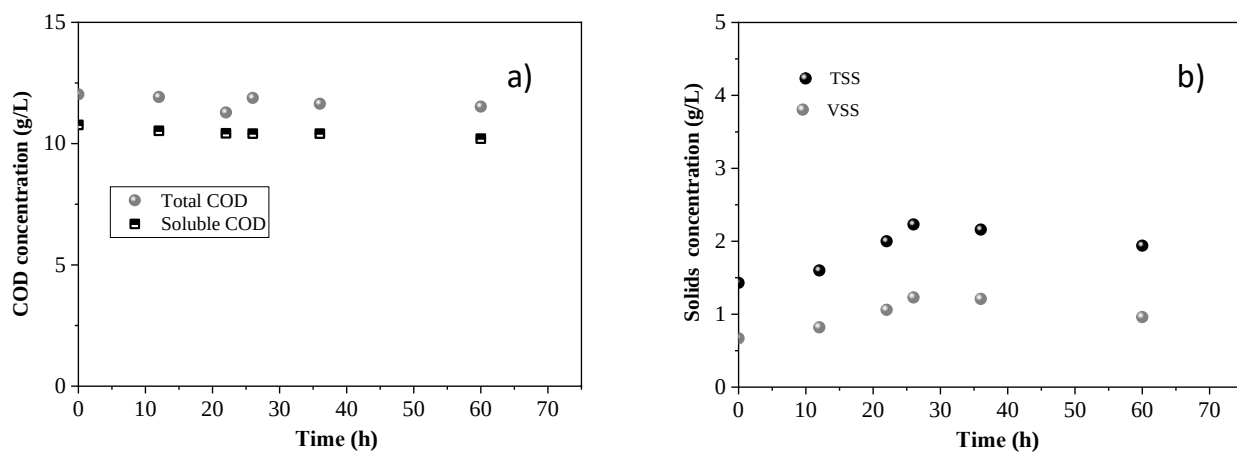


Figure 4. Soluble and Total COD concentration (a) and TSS and VSS (b) in batch1.

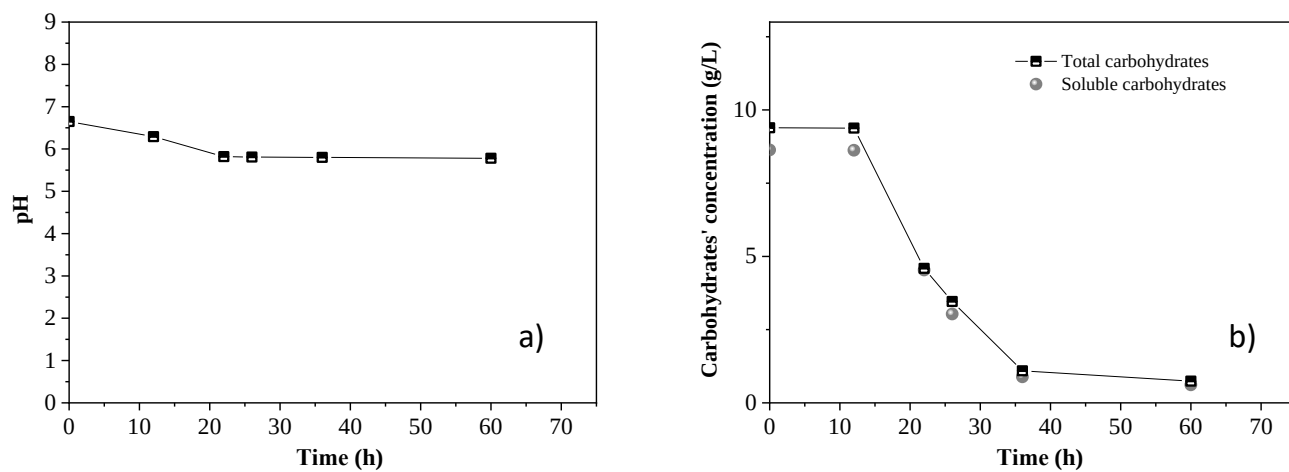


Figure 5. pH (a) and Soluble and Total carbohydrates in batch1.

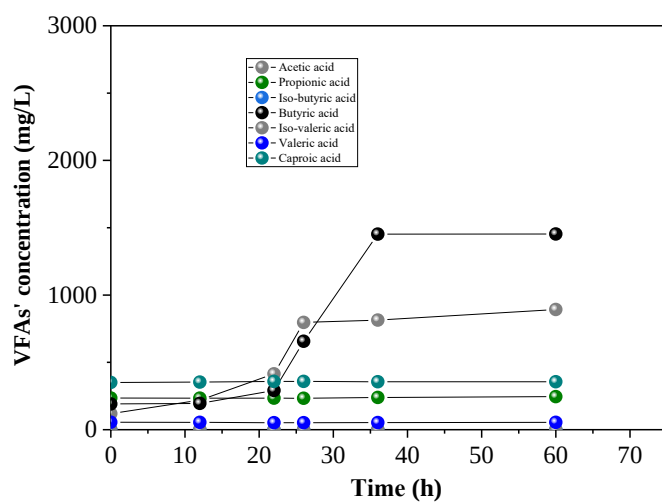


Figure 6. Volatile fatty acids (VFAs) produced in batch1.

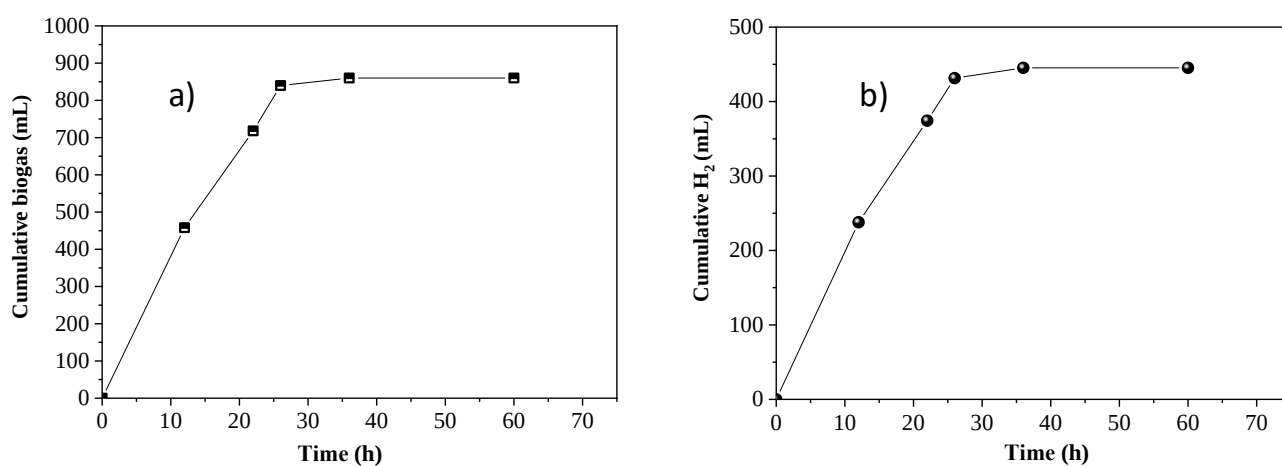


Figure 7. Cumulative biogas (a) and hydrogen production (b) in batch2.

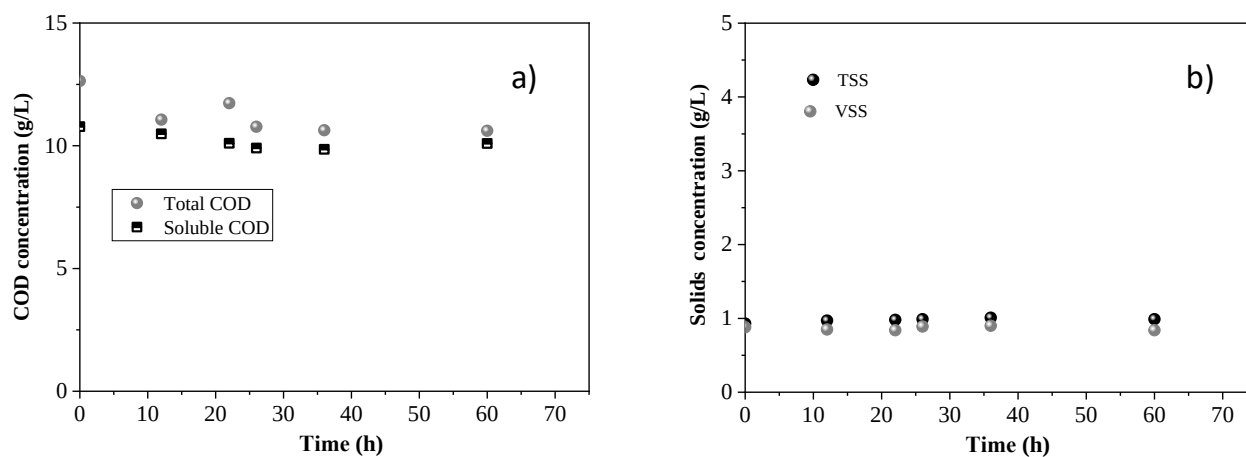


Figure 8. Soluble and Total COD concentration (a) and TSS and VSS (b) in batch2.

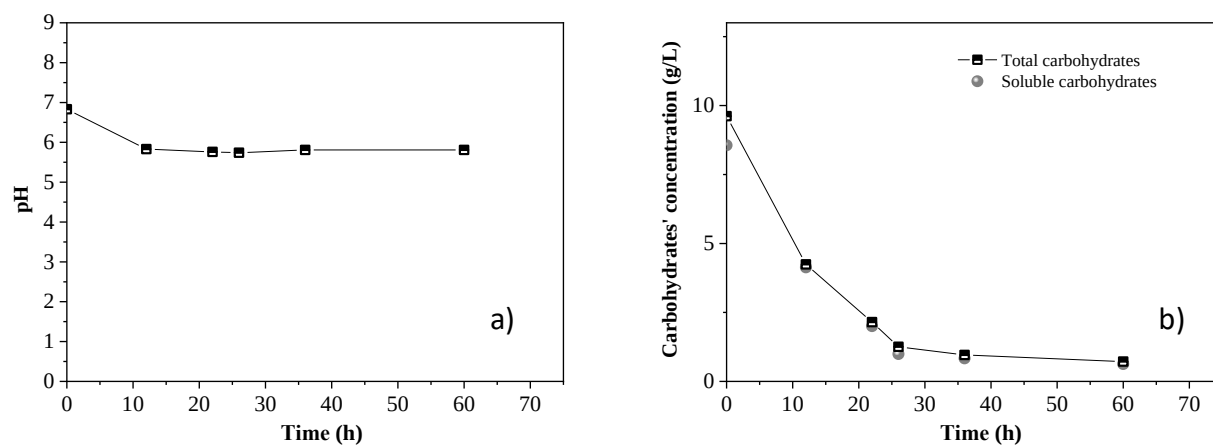


Figure 9. pH (a) and Soluble and Total carbohydrates in batch2



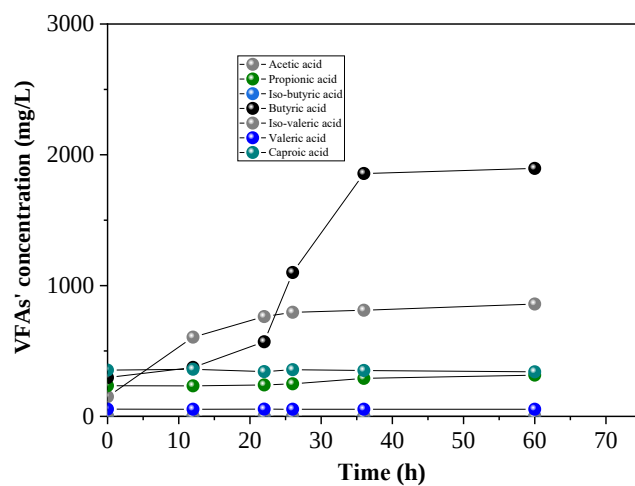


Figure 10. VFAs produced in batch2

Estimating the hydrogen production yield in both reactors, it can be reported that in batch 1, 0.42 mol H₂ /mol consumed sugars were produced, whereas in batch 2, the respective yield was 0.61 mol H₂ /mol consumed sugars. This indicates that the online pH-controlling approach led to a higher hydrogen yield.



SYMSITES

High Value New Resources validation

Deliverable name:High Value New Resources validation	
ANNEX	D4.4 _ANNEX IV _AITECH _GERM _Validation of Ectoine Properties and Associated Processes

Content

IV(a) Development of Ectoine-Based Cosmetic Formulations	2
IV(b) In Vivo Efficacy Assessment of the Ectoine Serum (External Studies).....	8
IV(c) Anti-Irritation Efficacy of the Ectoine Facial Gel	15
IV(d) Validation of Reactor Cleaning Using Treated Wastewater	21



IV(a) Development of Ectoine-Based Cosmetic Formulations

From methane-rich biogas generated through anaerobic digestion, it is possible to produce a valuable compound for the cosmetic industry, ectoin. This ingredient was discovered in extremely halophilic bacteria that protect themselves against osmotic stress and dehydration at high temperatures.

Ectoine, a naturally derived active ingredient, is an extremolyte as it protects the cellular function of microorganisms living in extreme environments with high salt concentrations by accumulating water inside the cells to protect them from salt-induced dehydration.

At the molecular level, this compound binds to water, creating complexes that surround proteins, lipids, etc. In this way, it forms and stabilizes protective hydration barriers around them. Ectoine acts similarly at the dermal level; in the following figure, we can observe how the skin barrier is strengthened, thus preventing the entry of free radicals and UV radiation, and reducing transepidermal water loss.

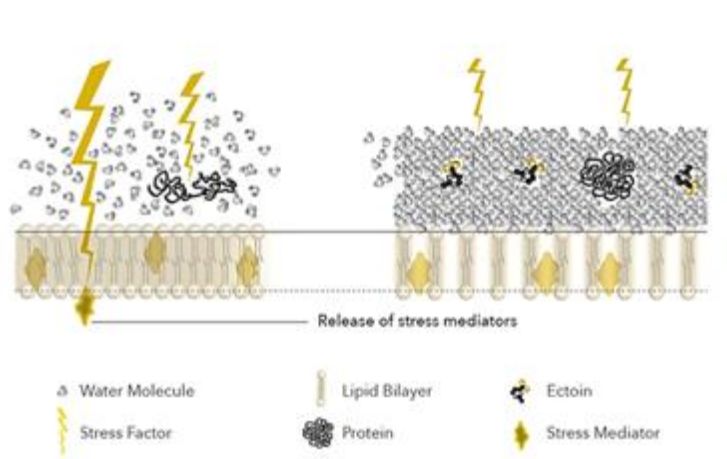


Figure 1. Effect of ectoine on the skin's dermal barrier

Ectoin helps maintain the skin's hydration equilibrium, keeping the barrier intact and strong. As a result, irritants stay out, moisture is locked in, and the skin is less likely to experience inflammation.

The same attributes that make ectoin such a benefit to the moisture barrier also help it to ward off environmental stressors like pollution and UV light. Ectoin prevents free radicals from penetrating the skin, reducing the damage to collagen and elastin fibers.

With the aim of demonstrating the symbiosis with the Cosmetic Industry (U-I) and obtaining high-value new resources (ectoine) from the biogas stream generated at the Ecosite, Aitex, with the help of Germaine, has carried out a series of activities to validate the properties of ectoine.

Development of serum formula at laboratory scale

A serum formula with ectoine has been developed at laboratory scale. The following figure shows the breakdown of ingredients for the serum (SE_2_Ectoine).

Table 1. Breakdown of ingredients for the SE_2 Ectoine formula.

INGREDIENT	INCI NAME	% IN FORMULA
Deionized Water	Aqua	87,04

INGREDIENT	INCI NAME	% IN FORMULA
Glycerin	Glycerin	8,00
Xanthan Gum	Xanthan Gum	1,00
Ectoin	Ectoin	1,00
High molecular weight hyaluronic acid	Sodium Hyaluronate	0,50
Low molecular weight hyaluronic acid	Sodium Hyaluronate	0,20
Tween 20	Polysorbate 20	0,70
Parfum	-	0,46
Sensicare C 1000	Phenoxyethanol, glyceryl laurate	1,00
NaOH 50%	Sodium Hydroxide, Aqua	0,10

Bioburden test, challenge test and patch test (external company)

An external company has collaborated on the bioburden test, challenge test, and patch test.

In collaboration with the external company, a bioburden test was conducted to check if the sample was contaminated during the manufacturing process and if the samples comply with the established limits for microbiological control of cosmetics according to the EN ISO 17516:2014 standard. The following table shows the results obtained:

Table 2. Results obtained from the Bioburden test for the SE_2 Ectoine sample.

Microorganismo Microorganism	Norma Standard	Resultado de la Detección del Microorganismo en el tamaño seleccionado de la muestra Result of Detection of the microorganism in the selected size of the sample
<i>Staphylococcus aureus</i>	EN ISO 22718:2015/A1:2022	Ausencia Absence
<i>Escherichia coli</i>	EN ISO 21150:2015/A1:2022	Ausencia Absence
<i>Pseudomonas aeruginosa</i>	EN ISO 22717:2015/A1:2022	Ausencia Absence
<i>Candida albicans</i>	EN ISO 18416:2015/A1:2022	Ausencia Absence
Ensayo de idoneidad Suitability test		Conforme Compliant

On the other hand, the Challenge test was carried out over 28 days to evaluate the efficacy of the preservative, with positive results.

Table 3. Results obtained from the Challenge test for the SE_2 Ectoïne sample.

Cepas referencia Strains reference	Inóculo Inoculum (ufc/mL) (Vol.0.2 mL)	7 días 7 days (ufc/mL)	14 días 14 days (ufc/mL)	28 días 28 days (ufc/mL)
Staphylococcus aureus (ATCC 6538)	N: 3,30E+07 N ₅ : 3,30E+05 IgN ₅ : 5,52	N: < 100 IgN ₅ : 2,00 R _x : 3,52	N: < 100 IgN ₅ : 2,00 R _x : 3,52	N: < 100 IgN ₅ : 2,00 R _x : 3,52
Escherichia coli (ATCC 8739)	N: 1,90E+07 N ₅ : 1,90E+05 IgN ₅ : 5,28	N: < 100 IgN ₅ : 2,00 R _x : 3,28	N: < 100 IgN ₅ : 2,00 R _x : 3,28	N: < 100 IgN ₅ : 2,00 R _x : 3,28
Pseudomonas aeruginosa (ATCC 9027)	N: 5,60E+07 N ₅ : 5,60E+05 IgN ₅ : 5,75	N: < 100 IgN ₅ : 2,00 R _x : 3,75	N: < 100 IgN ₅ : 2,00 R _x : 3,75	N: < 100 IgN ₅ : 2,00 R _x : 3,75
Candida albicans (ATCC 10231)	N: 3,90E+06 N ₅ : 3,90E+04 IgN ₅ : 4,59	N: < 100 IgN ₅ : 2,00 R _x : 2,6	N: < 100 IgN ₅ : 2,00 R _x : 2,6	N: < 100 IgN ₅ : 2,00 R _x : 2,6
Aspergillus brasiliensis (ATCC 16404)	N: 2,90E+06 N ₅ : 2,90E+04 IgN ₅ : 4,46	N: --- IgN ₅ : --- R _x : ---	N: < 100 IgN ₅ : 2,00 R _x : 2,47	N: < 100 IgN ₅ : 2,00 R _x : 2,47

Finally, the external company has collaborated in conducting a patch test to verify the skin compatibility of the cosmetic product on 10 volunteers after a single application on the skin and under an occlusive patch for 48 hours, monitored by a dermatologist. Based on the results obtained under the experimental conditions adopted, it is confirmed that the product has 'VERY GOOD SKIN COMPATIBILITY.'

Stability study

A stability study of the cosmetic product was carried out over 2 months at room temperature and in a climate chamber at 45°C. During this period, parameters such as viscosity were measured, and no significant changes were observed. Additionally, the texture, smell, and color of the serum were monitored during this period. In the following image, it can be seen that the formulas in the climate chamber acquired a yellowish color 2 months after manufacturing, whereas at room temperature, they remained the same as at the beginning.



Figure 2. Stability check of formulas at 2 months (order from left to right) SE_2 Ectoïne C.C., SE_2 Ref C.C., SE_2 Ectoïne at room temperature, and SE_2 Ref at room temperature

Development of serum formula at semi-industrial scale

The serum with ectoine, previously developed at laboratory scale, has been manufactured in a 50 kg reactor to evaluate the conditions of the procedure and the behavior of the formula in said reactor.

Below are images taken during the scaling process. In the image on the left, the reactor screen can be seen during manufacturing, and in the image on the right, the 50 kg reactor is shown





Figure 3. Reactor screen during scaling (left image) and reactor during manufacturing (right image).

When the product is completely homogenized, it is discharged into drums and then packaged.



Figure 4. Discharge of serum into drums (left image) packaging of the serum with ectoine (right image).

It should be noted that if the product is not too viscous, it can be discharged manually without the need to pressurize the system. However, in this case, it was necessary to activate the pressure during discharge due to the high viscosity of the product.

Study of the rheological behavior of the serum

A study of the rheological behavior of the serum manufactured with ectoine in the reactor has been conducted.

The following table shows the data obtained from the rheology study conducted for the SE_2_LOTE 3 serum at different shear rates, using spindle 5 with the Brookfield viscometer. It is noteworthy that the laboratory temperature was 21.0 °C. It is important to consider this factor as it significantly influences the viscosity value.

Table 4. Viscosity measurement of SE_2_LOTE 3 formula at different shear rates using spindle 5.

Spindle	Spindle speed (rpm)	Viscosity (cP)
5	5	13840
	10	7680



Spindle	Spindle speed (rpm)	Viscosity (cP)
	20	4360
	30	3027
	50	1928
	70	1583
	100	1184

The viscosity data is graphically represented to observe its behavior against shear rate. As shown in the following graph, as the shear rate increases, the viscosity of the product decreases.

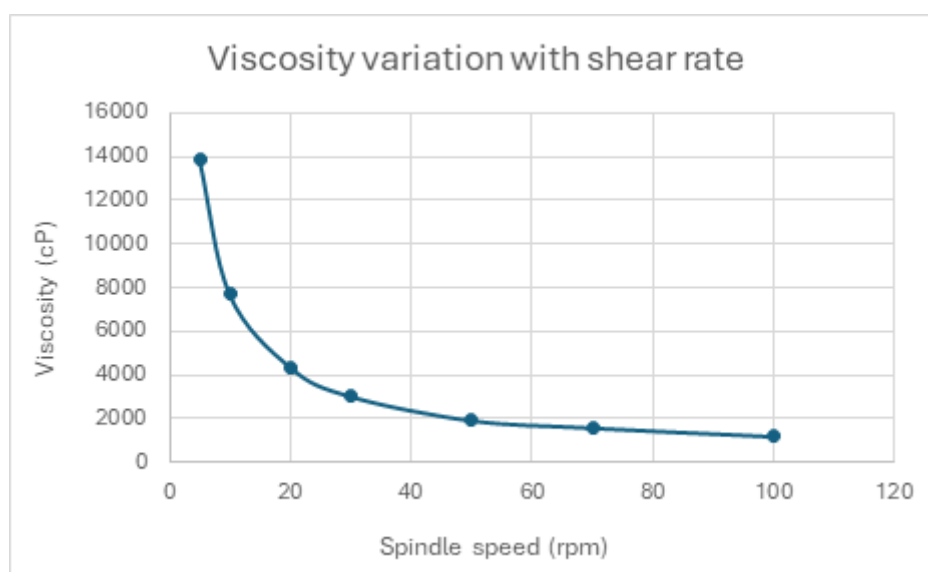


Figure 5. Variation of viscosity versus shear rate of formula SE_2_LOT 3.

This is a pseudoplastic behavior because the viscosity decreases as the shear rate increases. These are materials whose viscosity is reduced when the deformation rate increases. Many materials exhibit this type of behavior. For example, numerous substances found in emulsions, suspensions, or dispersions are examples of this type of fluid.

Evaluation of Natural Origin Content (ISO 16128)

The serum developed with ectoine has been evaluated for its percentage of natural origin ingredients in accordance with UNE-EN-ISO 16128, following both Part 1 (Guidelines on technical definitions and criteria for natural and organic cosmetic ingredients and products) and Part 2 (Criteria for ingredients and products). This assessment was carried out by considering the origin and processing method of each ingredient in the formulation, in line with the criteria established in the standard. As a result of this evaluation, **the product contains 97.63% ingredients of natural origin**, calculated in accordance with the methodology and requirements set out in **UNE-EN-ISO 16128**.

Antioxidant Capacity Assay

The antioxidant capacity of the following extracts studied within the Symsites project was evaluated:

- Ectoine
- High-molecular-weight hyaluronic acid
- Low-molecular-weight hyaluronic acid

The antioxidant capacity of these extracts was determined using a colorimetric assay based on the reduction of Cu^{2+} to Cu^{+} , a method that allows the quantification of the total antioxidant activity of a



sample. The reduction of the cupric ion is a consequence of the presence of compounds with electron-donating capacity, which results in an increase in the absorbance of the formed complex, measured by spectrophotometry. Results are expressed as Trolox equivalents (TAC), using this synthetic antioxidant as a reference standard.

For the assay, solutions of each extract were prepared at concentrations of 0.25%, 0.5%, and 1%, including in all cases a blank corresponding to the solvent used. As a positive control of the method, solutions of ascorbic acid (vitamin C) were used, within a concentration range of 50 to 200 µg/mL, in order to verify the correct performance of the assay and the sensitivity of the system.

Once the solutions were prepared, the corresponding volume of sample together with the specific reagents of the assay kit was added to each well of the microplate. After the incubation time indicated in the protocol, the plate was placed in the microplate reader and absorbance was measured at 570 nm.

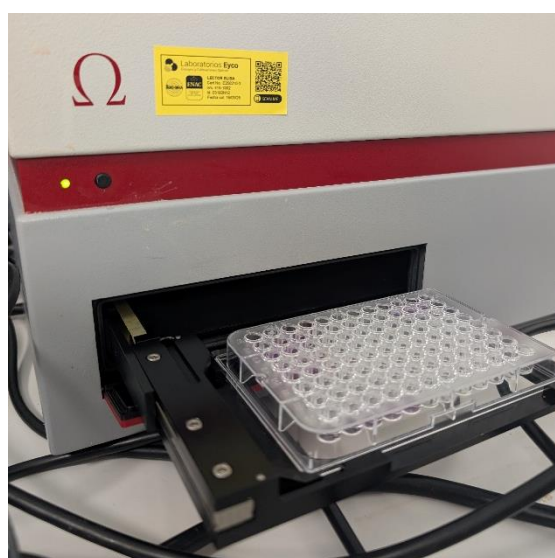


Figure 6. Plate reader used for absorbance determination at 570 nm.

In parallel, a standard calibration curve was constructed using Trolox at different known concentrations, making it possible to establish the linear relationship between absorbance and antioxidant concentration. From this curve, the slope was obtained and used to calculate the total antioxidant capacity of the samples.

Once the absorbance values were recorded, the total antioxidant capacity (TAC) was calculated using the following equation:

$$TAC(\mu M) = \frac{(A_{570})_{\text{sample}} - (A_{570})_{\text{blank}}}{\text{Slope}(\mu M^{-1})} \cdot n$$

where n corresponds to the dilution factor of the sample

The absorbance values obtained for the tested extracts were very low, in some cases even lower than those of the blank, resulting in negative TAC values. This behavior indicates that the evaluated extracts do not show a detectable antioxidant capacity using this method within the tested concentration range.



These results show that both ectoine and hyaluronic acids, regardless of their molecular weight, do not act as direct antioxidants in this type of chemical assay. This finding is consistent with the primary function described for these compounds, which is mainly associated with moisturizing, osmoprotective, and cellular stabilization properties rather than with direct antioxidant activity based on radical scavenging.

Consequently, under the evaluated conditions, the analyzed extracts can be considered to lack significant antioxidant capacity.

IV(b) In Vivo Efficacy Assessment of the Ectoine Serum (External Studies)

Efficacy test

Externally, in vivo tests have been conducted using a multi-probe micro caya system, involving 21 volunteers. Below are the probes used in the study to measure moisturizing efficacy, transepidermal water loss, and improvement in skin elasticity and firmness:

- **Corneometer® CM 825:** Surface hydration
- **Tewameter® TM 300:** transepidermal water loss
- **Cutometer® dual MPA 580:** elasticity and firmness

The properties of ectoin have been validated by AITEX, demonstrating statistically significant improvements in:

- **Hydration**
- **Firmness**
- **Elasticity**

Below, the calculations and statistics carried out are shown.

Hydration

The following table shows the average hydration values obtained at time 0 and at 28 days, as well as the percentage increase in hydration.

Table 5. Average hydration values and percentage increase in hydration.

Hydration t_0	Hydration t_f	% Increase Hyd
48,00	56,21	18,93

A statistical analysis has been performed using Jamovi software. First, descriptive statistical results were obtained by applying the Shapiro-Wilk test.

The following table shows the obtained descriptive values and the Shapiro-Wilk test results:





Descriptives		
	Hydration t0	Hydration tf
N	21	21
Mean	48.0	56.2
Median	46.0	55.8
Standard deviation	7.38	12.2
Minimum	35.8	34.5
Maximum	67.0	98.0
Shapiro-Wilk W	0.938	0.830
Shapiro-Wilk p	0.200	0.002

The obtained p-value is below 0,05, therefore, the data doesn't follow a normal distribution. Since the p-value is <0.05 , the Wilcoxon test has been applied. The following table shows the results obtained with this test.

Paired Samples T-Test				
			Statistic	p
Hydration t0	Hydration tf	Wilcoxon W	42.0	0.005

Note. $H_0: \mu \text{ Measure 1} - \text{Measure 2} < 0$

Since the p-value is <0.05 , it can be concluded that there is a significant increase in hydration after using the serum for 28 days.

Firmness

With the application of this serum, a significant reduction in penetration is obtained throughout the study, thus validating the significant improvement effect in the increase of firmness provided by the serum after its application for 28 days.

Table 6. Average penetration values and percentage reduction penetration

Penetration (mm) t_0	Penetration (mm) t_f	%Reduc Penetration (mm)
0,368	0,239	-28,36

Below, descriptives and Shapiro-Wilk test are shown:

Descriptives		
	Penetr (mm) tf izq	Penetr (mm) t0 izq
N	21	21
Mean	0.239	0.368
Median	0.233	0.399
Standard deviation	0.0775	0.111
Shapiro-Wilk W	0.945	0.914
Shapiro-Wilk p	0.269	0.066

According to Shapiro-Wilk test the data follow a normal distribution ($p>0,05$) and so a Student T test is applied to compare the means.





Paired Samples T-Test

			statistic	df	p
Penetr (mm) t0 izq	Penetr (mm) tf izq	Student's t	5.22	20.0	< .001

Note. $H_a: \mu_{\text{Measure 1}} - \mu_{\text{Measure 2}} \neq 0$

Since the p value is <0.05, it can be concluded that there is a significant increase in firmness after using the serum for 28 days.

Elasticity

In this last case, the following table shows the average skin recovery at t_0 and t_f , where there is an increase in elasticity.

Table 7. Average recovery values and percentage increase in elasticity.

% recovery t_0	% recovery t_f	% increase
52,719	73,59	42,24

Below, descriptives and Shapiro-Wilk test are shown:

Descriptives

	% recuper t0 izq	% recuper tf izq
N	21	21
Mean	52.7	73.6
Median	52.9	76.4
Standard deviation	12.7	14.4
Shapiro-Wilk W	0.971	0.932
Shapiro-Wilk p	0.765	0.154

According to Shapiro-Wilk test the data follow a normal distribution ($p > 0.05$) and so a Student T test is applied to compare the means.

Paired Samples T-Test

			statistic	df	p
% recuper t0 izq	% recuper tf izq	Student's t	-9.76	20.0	< .001

Note. $H_a: \mu_{\text{Measure 1}} - \mu_{\text{Measure 2}} \neq 0$

Since the p value is <0.05, it can be concluded that there is a significant increase in elasticity after using the serum for 28 days.

Repeat of the Efficacy Test

Since the efficacy test showed statistically significant differences when comparing the results obtained at baseline (T0) and after 28 days of application (T28), an effect of the treatment over the application period was confirmed. However, no statistically significant differences were observed between the ectoine-containing serum and the placebo serum, as the study was designed as an intrapersonal comparative test, with the ectoine serum applied to one side of the face and the



placebo serum to the contralateral side. In light of the lack of significant differences between both formulations, it was decided to repeat the efficacy study using a newly developed simplified formulation, composed exclusively of ectoine and high–molecular-weight hyaluronic acid, in order to more specifically evaluate the effect of ectoine and to facilitate the detection of potential statistically significant differences through statistical analysis.

The following figure shows the breakdown of ingredients for the serum (SE_2_BATCH 4).

Table 8. Breakdown of ingredients for the SE_2_BATCH 4 formula.

INGREDIENT	INCI NAME	% IN FORMULA
Deionized Water	Aqua	87,24
Glycerin	Glycerin	8,00
XanthanGum	Xanthan Gum	1,00
Ectoin	Ectoin	1,00
High molecular weight hyaluronic acid	Sodium Hyaluronate	0,50
Tween 20	Polysorbate20	0,70
Parfum	-	0,46
Sensicare C 1000	Phenoxyethanol, glyceryl laurate	1,00
NaOH 50%	SodiumHydroxide, Aqua	0,1

Transepidermal water loss (TEWL)

The following table shows the percentage change in transepidermal water loss (TEWL) after 28 days of treatment for the ectoine-treated side and the placebo-treated side.

Table 9. Percentage Change in Transepidermal Water Loss (TEWL)

Ectoine-treated side	Placebo-treated side
-19,19	-13,96

Transepidermal water loss (TEWL) measurements showed a reduction after 28 days of treatment on both facial sides. The ectoine-treated side exhibited a greater mean decrease in TEWL (–19.19%) compared to the placebo-treated side (–13.96%), indicating an improvement in skin barrier function. Although the difference between treatments did not reach statistical significance, the observed trend suggests a protective effect of ectoine against water loss.

Below, descriptives and Shapiro-Wilk test are shown:



Descriptives

Descriptives		
	%Reduc TEWL dcha	%Reduc TEWL izqda
N	21	21
Missing	0	0
Mean	-19.2	-14.0
Median	-16.6	-21.0
Standard deviation	14.2	26.6
Minimum	-51.4	-53.9
Maximum	4.35	43.0
Shapiro-Wilk W	0.972	0.939
Shapiro-Wilk p	0.778	0.209

Paired Samples T-Test

Paired Samples T-Test					
			statistic	df	p
%Reduc TEWL dcha	%Reduc TEWL izqda	Student's t	-0.969	20.0	0.344

Note. H₀: μ Measure 1 - Measure 2 = 0

Differences between the ectoine-treated and placebo-treated facial sides were first evaluated using the Wilcoxon signed-rank test, which showed no statistically significant differences ($p > 0.05$). Given that data normality was confirmed by the Shapiro – Wilk test ($p > 0.05$), a paired Student’ s t-test was subsequently applied, also revealing no significant differences between treatments ($p = 0.344$).

Hydration

The following table shows the percentage increase in facial skin hydration after 28 days of treatment for the ectoine-treated side and the placebo-treated side.

Table 10. Percentage Increase in Skin Hydration (Corneometer)

Ectoine-treated side	Placebo-treated side
20,32	-11,69

Statistical analysis of skin hydration data measured by corneometry showed a significantly higher mean increase on the ectoine-treated side (20.3%) compared to the placebo side (-11.7%). Since the data met the normality criterion (Shapiro–Wilk, $p > 0.05$), a paired Student’ s t-test was applied, revealing highly significant differences between treatments ($t = 5.14$; $p < 0.001$).



Descriptives

Descriptives

	%Aument Hid dcha	%Aument Hid izqda
N	21	21
Missing	0	0
Mean	20.3	-11.7
Median	24.6	-9.89
Standard deviation	25.7	21.1
Minimum	-28.7	-59.2
Maximum	77.2	18.9
Shapiro-Wilk W	0.977	0.957
Shapiro-Wilk p	0.869	0.466

Paired Samples T-Test

Paired Samples T-Test

			statistic	df	p
%Aument Hid dcha	%Aument Hid izqda	Student's t	5.14	20.0	< .001

Note. $H_0: \mu \text{ Measure 1} - \text{Measure 2} = 0$

Firmness

The following table shows the percentage reduction in skin penetration measured by cutometry after 28 days of treatment for the ectoine-treated side and the placebo-treated side.

Table 11. Percentage reduction in Skin Penetration (Cutometer)

Ectoine-treated side	Placebo-treated side)
-1,00	-1,51



Descriptives

Descriptives

	%Reduc Penetr dcha	%Reduc Penetr izqda
N	21	21
Missing	0	0
Mean	-0.995	-1.51
Median	-1.35	-3.31
Standard deviation	8.54	9.89
Minimum	-18.6	-18.4
Maximum	11.4	15.5
Shapiro-Wilk W	0.965	0.953
Shapiro-Wilk p	0.613	0.391

Paired Samples T-Test

Paired Samples T-Test

			statistic	df	p
%Reduc Penetr dcha	%Reduc Penetr izqda	Student's t	0.152	20.0	0.881

Note. $H_0: \mu \text{ Measure 1} - \text{Measure 2} = 0$

Statistical analysis of skin firmness assessed by cutometry (% reduction in penetration) showed no statistically significant differences between the ectoine-treated side and the placebo side. After confirming data normality (Shapiro–Wilk, $p > 0.05$), a paired Student's t-test was applied, revealing no significant differences between treatments ($t = 0.152$; $p = 0.881$).

Elasticity

The following table shows the percentage increase in skin elasticity measured by cutometry after 28 days of treatment for the ectoine-treated side and the placebo-treated side.

Table 12. Percentage Increase in Skin Elasticity (Cutometer)

Ectoine-treated side	Placebo-treated side
-5.43	0.86



Descriptives

Descriptives		
	%Aumento dcha	%Aumento izqda
N	21	21
Missing	0	0
Mean	-5.43	0.863
Median	-7.64	7.62
Standard deviation	13.1	21.4
Minimum	-32.5	-38.5
Maximum	14.7	30.3
Shapiro-Wilk W	0.965	0.921
Shapiro-Wilk p	0.629	0.090

Paired Samples T-Test

Paired Samples T-Test					
			statistic	df	p
%Aumento dcha	%Aumento izqda	Student's t	-1.25	20.0	0.226

Note. H₀: $\mu_{\text{Measure 1}} - \mu_{\text{Measure 2}} = 0$

Statistical analysis of skin elasticity assessed by cutometry (% increase) showed no statistically significant differences between the ectoine-treated side and the placebo side. After confirming data normality (Shapiro–Wilk, $p > 0.05$), a paired Student's t-test was applied, revealing no significant differences between treatments ($t = -1.25$; $p = 0.226$).

IV(c) Anti-Irritation Efficacy of the Ectoine Facial Gel

FACIAL GEL

Within the framework of the project, an ectoine-containing facial gel was developed with the aim of evaluating the ability of this active ingredient to reduce skin irritation induced by the surfactants present in the formulation. Prior to the development of the final formulation, a preformulation stage was carried out in which different rheological modifiers were evaluated in order to optimize the texture, stability, and rheological behavior of the system. As a result of this comparative evaluation, Carbopol® U10 was selected as the most suitable rheological modifier for the development of the base gel.

In the following figure, the increase in viscosity observed in the formulation after the incorporation of Carbopol® U10 is shown (left image). The image on the right corresponds to the surfactant base in the absence of the carbomer. In order to assess the compatibility between the carbomer and the surfactant system, different proportions of Carbopol® U10 and surfactants were tested, as in some preliminary formulations a significant reduction, or even a complete loss, of viscosity was observed after the addition of the surfactants to the gelified base. Based on these comparative trials, the most



appropriate ratio was selected, in which no relevant decrease in viscosity occurred, thus ensuring stable rheological properties in the final formulation.

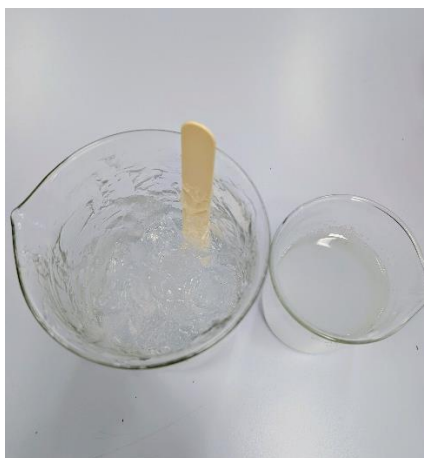


Figure 7. Effect of Carbopol U10 and the surfactant system on the viscosity of the formulation.

After selecting the rheological modifier, the development of the ectoine-containing facial gel formulation was carried out, defining the qualitative and quantitative composition of the final product. The formulation was adjusted with the aim of ensuring adequate physicochemical stability and proper incorporation of the active ingredient. The following table presents the final facial gel formulation developed within the framework of the project.

Table 13. Ingredient composition and functions of the developed facial gel.

INGREDIENT	INCI name	FUNCTION
Base gel with 1,3% Carbopol U10	Carbomer	Thickener
	Aqua	
Sodium Lauroyl Methyl Isethionate	Sodium Lauroyl Methyl Isethionate	Surfactant
Cocamidopropyl Betaine	Cocamidopropyl Betaine	Surfactant
Coco Glucoside	Coco Glucoside	Surfactant
Glycerin	Glycerin	Humectant
Sensicare C1000	Phenoxyethanol (and) Glyceryl Laurate	Preservative
Niacinamide	Niacinamide	Active ingredient
Parfum	Parfum	Fragrance
Ectoin	Ectoin	Active ingredient

In the following figure, the reference facial gel (placebo) is shown on the left, and the ectoine-containing facial gel on the right, both developed in the laboratory.



Figure 8. Placebo facial gel (left) and ectoine-containing facial gel (right).

Viscosity

The viscosity of the developed formulations was determined by means of comparative rheological measurements between the base facial gel (reference) and the ectoine-containing facial gel, using the same test method. The results obtained show, in both cases, pseudoplastic behavior, characterized by a progressive decrease in viscosity as the shear rate increases. When comparing both formulations, viscosity values were found to be within very similar ranges throughout the entire analyzed shear rate interval, with no significant differences attributable to the incorporation of ectoine into the formulation. These results indicate that the addition of the active ingredient does not significantly modify the rheological profile of the gel, maintaining consistency and application properties equivalent to those of the reference gel.

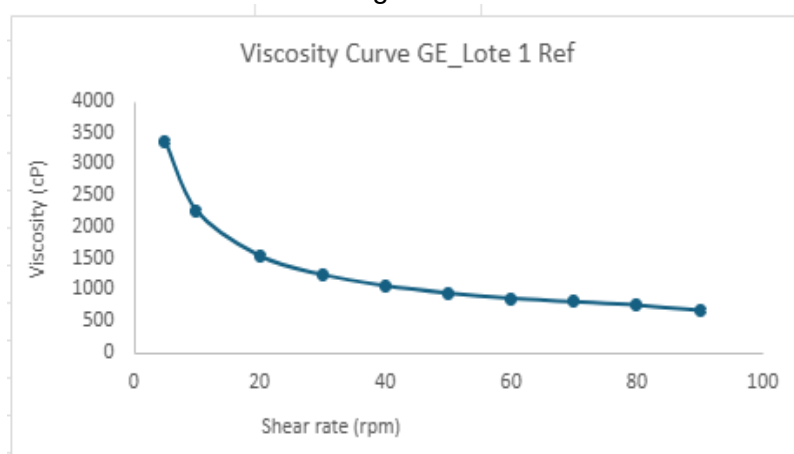


Figure 9. Placebo facial gel viscosity curve



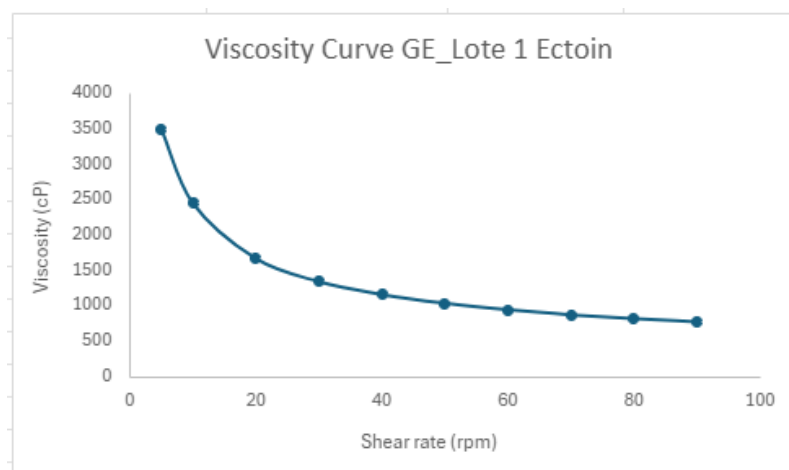


Figure 10. Ectoine-containing facial gel viscosity curve

Bioburden test and challenge test (external company)

An external company has collaborated on the bioburden test and challenge test.

In collaboration with the external company, a bioburden test was conducted to check if the sample was contaminated during the manufacturing process and if the samples comply with the established limits for microbiological control of cosmetics according to the EN ISO 17516:2014 standard. The following table shows the results obtained:

Table 14. Results obtained from the Bioburden test for the GE_Lote 1 Ectoin sample.

Microorganismo Microorganism	Norma Standard	Resultado de la Detección del Microorganismo en el tamaño seleccionado de la muestra Result of Detection of the microorganism in the selected size of the sample
<i>Staphylococcus aureus</i>	EN ISO 22718:2015/A1:2022	Ausencia Absence
<i>Escherichia coli</i>	EN ISO 21150:2015/A1:2022	Ausencia Absence
<i>Pseudomonas aeruginosa</i>	EN ISO 22717:2015/A1:2022	Ausencia Absence
<i>Candida albicans</i>	EN ISO 18416:2015/A1:2022	Ausencia Absence
Ensayo de idoneidad Suitability test		Conforme Compliant

On the other hand, the Challenge test was carried out over 28 days to evaluate the efficacy of the preservative, with positive results.

Table 15. Results obtained from the Challenge test for the GE_Lote 1 Ectoio sample.

Cepas referencia Strains reference	Inóculo Inoculum (ufc/mL) (Vol 0,2 mL)	7 días 7 days (ufc/mL)	14 días 14 days (ufc/mL)	28 días 28 days (ufc/mL)
<i>Staphylococcus aureus</i> (CECT 239 = ATCC 6538)	N: 7,20E+07 N ₀ : 7,20E+05 IgN ₀ : 5,86	N: < 100 IgN _x : 2,00 R _x : 3,86	N: < 100 IgN _x : 2,00 R _x : 3,86	N: < 100 IgN _x : 2,00 R _x : 3,86
<i>Escherichia coli</i> (CECT 516 = ATCC 8739)	N: 3,50E+07 N ₀ : 3,50E+05 IgN ₀ : 5,54	N: < 100 IgN _x : 2,00 R _x : 3,55	N: < 100 IgN _x : 2,00 R _x : 3,55	N: < 100 IgN _x : 2,00 R _x : 3,55
<i>Pseudomonas aeruginosa</i> (CECT 111 = ATCC 9027)	N: 6,30E+07 N ₀ : 6,30E+05 IgN ₀ : 5,80	N: < 100 IgN _x : 2,00 R _x : 3,8	N: < 100 IgN _x : 2,00 R _x : 3,8	N: < 100 IgN _x : 2,00 R _x : 3,8
<i>Candida albicans</i> (CECT 1394 = ATCC 10231)	N: 3,50E+06 N ₀ : 3,50E+04 IgN ₀ : 4,54	N: < 100 IgN _x : 2,00 R _x : 2,55	N: < 100 IgN _x : 2,00 R _x : 2,55	N: < 100 IgN _x : 2,00 R _x : 2,55
<i>Aspergillus brasiliensis</i> (CECT 2574 = ATCC 16404)	N: 2,70E+06 N ₀ : 2,70E+04 IgN ₀ : 4,43	N: --- IgN _x : --- R _x : ---	N: < 100 IgN _x : 2,00 R _x : 2,44	N: < 100 IgN _x : 2,00 R _x : 2,44

Stability study

A stability study of the facial gel was carried out over a period of 3 months, both at room temperature and under climatic chamber conditions at 40 °C. During this period, parameters such as viscosity were evaluated, and no significant variations were observed. In addition, the organoleptic properties of the product, including texture, odor, and color, were monitored. As shown in the following figure, no visual changes or abnormal color development were detected under any of the evaluated storage conditions, with the formulations remaining stable compared to their initial appearance.

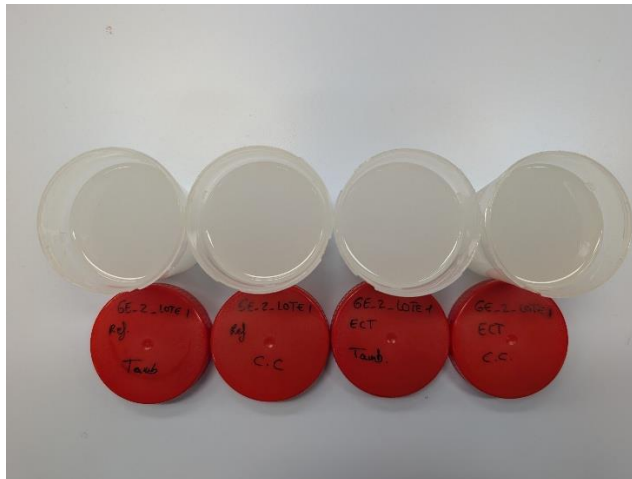


Figure 11. Stability check of formulas at 3 months (order from left to right) GE_2_Ref at room temperature, GE_2_Ref C.C., GE_2 Ectoio at room temperature, and GE_2 Ectoio C.C.

Ectoio Anti-Irritation Efficacy Validation Test

The objective of this study is to evaluate the efficacy of ectoine as an anti-irritant agent. To this end, an initial preliminary study was conducted externally with a reduced number of volunteers. Two types of facial gel were applied to each arm: one containing ectoine and one placebo. The effect was assessed by measuring transepidermal water loss (TEWL) using a Tewameter probe, in order to detect potential anti-irritant effects.

Two application modes were considered:

- **Occlusive** application in some volunteers.
- **Rinse-off** application in others, to simulate more realistic use under normal conditions.

Once the initial results are obtained, the most appropriate methodology will be selected to expand the study to a more representative sample.

Protocol for the Ectoine Anti-Irritation Efficacy Validation Test

Among the two application modes considered in the study, the *rinse-off* method was selected, as the evaluated product is a facial gel intended to be rinsed off after use. This approach is more representative than the occlusive method for simulating real conditions of application.

The procedure carried out through an external company was as follows: each arm was initially cleaned with 70% ethanol to remove residual grease and impurities. After drying, transepidermal water loss (TEWL) was measured at time 0. Given that the product is a facial gel, a frequency of two washes per day was established. For each wash, the area was pre-moistened with water, 3 mL of product were applied, the area was rubbed for 10 strokes, and finally rinsed with 150 mL of water.

As this is a facial cleansing product, the washing protocol led to an increase in transepidermal water loss (TEWL) in both arms as a result of the action of surfactants on the skin barrier. However, when comparing both treatments, the percentage increase in TEWL was lower in the arm treated with the ectoine facial gel (+39.8%) compared to the arm treated with the placebo (+54.3%). These results indicate that, although both products induce a washing-related alteration of the skin barrier, the ectoine gel significantly attenuates the impact of washing on barrier function, demonstrating a protective effect against surfactant-induced irritation.

Table 16. Percentage increase in TEWL following the washing protocol

Parameter	Ectoine facial gel (right arm)	Placebo facial gel (left arm)
% increase in TEWL (T2 vs T0)	+39,8 %	+54,3 %

Below, descriptives and Shapiro-Wilk test are shown:

Descriptives

Descriptives		
	%TEWL dcha	%TEWL IZQDA
N	21	21
Missing	0	0
Mean	39.8	54.3
Median	29.1	38.3
Standard deviation	45.0	49.9
Minimum	-23.1	-0.990
Maximum	182	205
Shapiro-Wilk W	0.874	0.815
Shapiro-Wilk p	0.011	0.001

According to Shapiro-Wilk test the data follow a normal distribution ($p > 0,05$) and so a Student T test is applied to compare the means.



Paired Samples T-Test

			statistic	df	p
%TEWL dcha	%TEWL IZQDA	Student's t	-1.43	20.0	0.168

Note. $H_0: \mu_{\text{Measure 1}} - \mu_{\text{Measure 2}} \neq 0$

Prior to the comparative analysis, the normality of the paired differences was assessed using the Shapiro–Wilk test, yielding p-values greater than 0.05, which allowed the assumption of a normal data distribution. Accordingly, a paired Student's t-test was applied to compare the percentage increase in TEWL between the two treatments. Although the ectoine facial gel showed a lower mean increase in TEWL compared to the placebo, the observed differences did not reach statistical significance ($p > 0.05$).

IV(d) Validation of Reactor Cleaning Using Treated Wastewater Reuse of Treated Wastewater from the Pilot Plant for Cosmetic Reactor Cleaning

- **Determination of the Optimal Percentage of Permeate Water**

A laboratory-scale study will be carried out to evaluate different combinations and dilutions of **permeate water from the pilot plant (Ecosite España)** after passing through the ultrafiltration (UF) membrane. The main objective is to determine the **optimal percentage of treated wastewater** suitable for reuse in the cleaning of cosmetic reactors.

To this end, the treated water will be combined with tap water at different dilution ratios, which will be evaluated in order to identify the most appropriate mixture. Physicochemical and microbiological parameters of the treated water and its dilutions will be analyzed, in accordance with the criteria established in the current legislation for cleaning water (**Royal Decree 1085/2024**). The following table shows the maximum admissible values for industrial use, except in food industry applications.

Table 17. Maximum admissible value for industrial use, except in the food industry

Uso industrial, excepto en la empresa alimentaria	<i>E. coli</i> (UFC/100 mL)	Turbidez (UNT)	SS (mg/L)	<i>Legionella spp.</i> (UFC/L)	Contaminantes
Calidad I. A+. – Torres de refrigeración y condensadores evaporativos en agua de aporte a la torre.	Ausencia	5	10	Ver observaciones	Ver observaciones
Calidad I. C. – Aguas de proceso: aquellas que están en contacto directo en algún momento con materias primas, materiales, productos intermedios o productos finales de un proceso industrial. – Aguas de limpieza: aguas destinadas a la limpieza de equipos, objetos, materiales, tuberías y superficies en áreas industriales, excepto en la empresa alimentaria. – Otros usos industriales.	1.000	–	Conforme DARU	Ver observaciones	Ver observaciones

- **Reuse of Treated Wastewater for Reactor Cleaning**

In this task, the feasibility of reusing treated water from the pilot plant at an industrial scale will be evaluated. This water will be used in the first rinse of an industrial-scale reactor employed in the manufacture of a cosmetic product, prior to carrying out the standard cleaning procedure using a CIP system.

The objective of this study is to validate the results obtained at laboratory scale and to ensure the effectiveness of the treated water in industrial applications, thereby guaranteeing its feasibility and



safety for use in the cosmetic industry. It should be noted that the reuse of treated water will first be assessed at laboratory scale (15 kg reactor) and subsequently in a 50 kg reactor.

To this end, the following activities will be carried out:

- After the manufacture of a cosmetic product, the selected treated water will be used to perform the first rinse of the reactor, and the efficiency of removal of product residues will be evaluated.
- Once the first rinse has been completed and after applying a standard CIP cleaning system, strategic points inside the reactor will be defined for sample collection using swabs.
- The collected samples will be analyzed to verify that microbiological parameters comply with the limits established by Royal Decree 1085/2024, which regulates water reuse for industrial purposes. This analysis will ensure that the treated water is safe for use in the cosmetic industry.

For sample analysis, a **bioburden test** will be requested from the Microbiology Department, including total and specific microbial counts of the reactor surface using swabs at strategic locations. The following bacteria and fungi must be analyzed in cosmetic applications:

- *E.Coli* → Bacteria
- *Pseudomonas* → Bacteria
- *Staphylococcus aureus* → Bacteria
- *Aspergillus* → Fungus
- *Candida albicans* → Fungus
- *Burkholderia* → Bacteria

The total microbial count must be **≤1000 colony-forming units (CFU)** to be considered free of microbiological contamination. It should be noted that, even if *Burkholderia* is not specifically selected for analysis, the total bacterial count quantifies all bacterial genera.

Based on the results obtained, the effectiveness of the previously selected optimal percentage of treated wastewater will be validated for its reuse in the first reactor rinse following cosmetic product manufacturing. These results will confirm the feasibility of its application in industrial cleaning processes within the cosmetic sector, ensuring both efficacy and safety.

Water is the most widely used raw material in the manufacture of cosmetic products and, in many cases, is the source of microbial contamination. Manufacturing water must have a defined quality, specifically physicochemical specifications that ensure the quality and stability of cosmetic products, as well as microbiological specifications. As a recommendation, these specifications should be at least equivalent to those required by the European Pharmacopoeia for purified water or by the **UNE-EN ISO 17516** standard with regard to microbiological limits for higher-risk products (those intended for children under 3 years of age, the eye area, or mucous membranes).

Regarding the quality of the water used for cleaning purposes, it should meet, as a minimum, the specifications established for water intended for human consumption (**Royal Decree 140/2003 of 7 February**, which establishes the health criteria for the quality of water intended for human consumption).



When a water treatment system is available, the equipment involved may vary mainly depending on the quality of the incoming water and the quality required for its intended use. In any case, the objective of water treatment is to remove as many existing contaminants as possible, such as suspended particles, cations, anions, and microorganisms.

There are different water treatment systems that allow water quality to be maintained. The most common include:

- **Physicochemical treatments:** ion exchange, electrodeionization, and reverse osmosis.
- **Microbiological treatments:** ozonation, chlorine and its derivatives, heat treatment (65–80 ° C), ultraviolet radiation, and filtration.
- **Validation of the Cleaning and Disinfection Process**

In this section, the reactor cleaning process for the reuse of treated wastewater is established, using permeate water from the Ecosite España pilot plant as the initial rinse water. Subsequently, a cleaning step with an alkaline solution is carried out, followed by a rinsing step and then a cleaning step with an acidic solution. The solutions used are shown in the following figure.

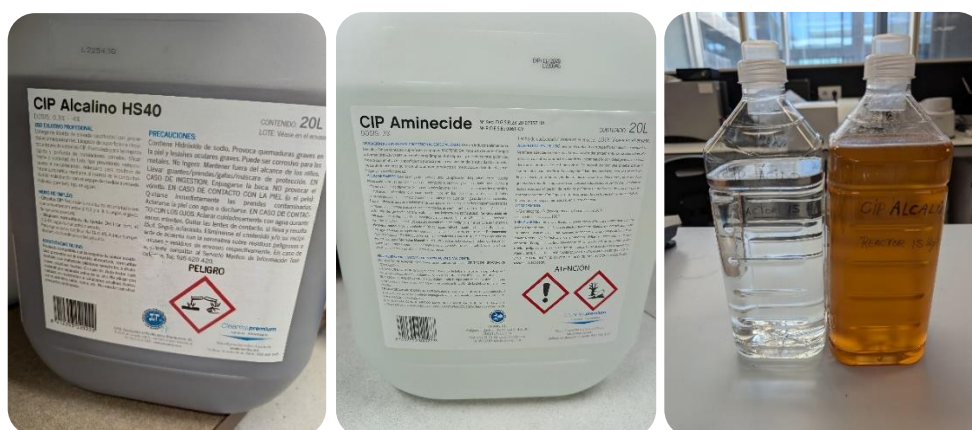


Figure 12. Alkaline (left) and acidic (center) CIP solutions used in the reactor cleaning process for wastewater reuse.

First, an initial cleaning of the reactor was carried out using permeate water from the pilot plant as the first rinse, with a 70% degreasing level. As the reactor has a capacity of 15 kg, a proportional amount of permeate water from Ecosite España was used, applying standard agitation and emulsification conditions. After this first rinse, a sample of the washing water was collected for hardness determination and for general and specific microbiological analysis. In addition, the microbiological status of the reactor surface was assessed by taking swab samples from different internal areas considered susceptible to contamination.

Subsequently, the reactor was disinfected following a sequential chemical cleaning protocol. First, a 2% sodium hydroxide solution heated to 70 ° C was applied under controlled agitation and emulsification conditions, after which the solution was discarded. This step was followed by a rinse with distilled water and then by treatment with a 3% acidic solution with biocidal activity. During this



stage, samples of the cleaning water were collected and swabs were taken from the internal surface of the reactor for microbiological analysis.

Finally, a final rinse was performed using distilled water. After this step, washing water samples were collected for physicochemical and microbiological analysis, together with swab samples from different reactor surfaces, in order to confirm the effectiveness of the cleaning and disinfection procedure. Representative photographs taken during the trials are shown in the following figure.



Figure 13. Photographs taken during the reactor cleaning validation test using treated wastewater.

Based on the samples collected after each washing step, the following parameters were evaluated. The data obtained are shown on the table below.

Table 18. Physicochemical parameters of water samples after each washing step.

Sample	pH	Conductivity ($\mu\text{S}/\text{cm}$)	Hardness (mg/L)	Appearance
Permeate water	7,20	2620	382	Yellowish
Cleaning 1 (permeate water)	7,34	2480	378	Yellowish
Cleaning 2 (base–acid)	10,20	1128	< 2	Colorless
Cleaning 3 (rinse water)	7,75	3,27	< 2	Colorless



As observed in the table, the results obtained after Cleaning 1 differ considerably from those obtained after Cleanings 2 and 3. Each parameter is evaluated below:

- **pH:** The pH remained relatively stable in the permeate water and rinsing samples, but increased significantly during the alkaline–acid cleaning step, which is expected due to the use of products such as sodium hydroxide.
- **Conductivity:** Conductivity decreased markedly after the alkaline–acid cleaning and even further after the rinsing step, indicating a reduction in the amount of dissolved ions.
- **Hardness:** Water hardness also decreased substantially after the alkaline–acid cleaning and subsequent rinsing, suggesting effective removal of minerals.
- **Appearance:** The appearance changed from yellowish to colorless, indicating an improvement in the visual cleanliness of the reactor.

In the following figure, the cleaning water samples collected after each washing step are shown. The container corresponding to Cleaning 1 (left image) clearly exhibits a yellowish coloration.

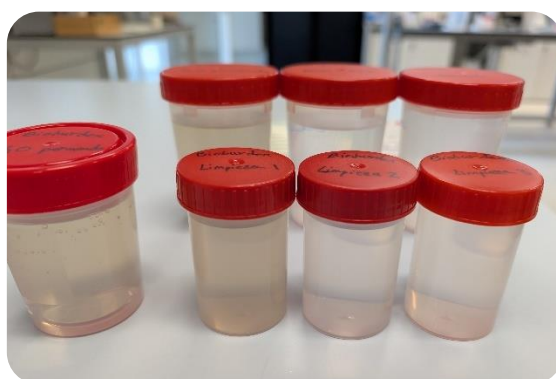


Figure 14. Cleaning water samples collected after each wash.

The results suggest that the alkaline–acid cleaning followed by a water rinse was effective in reducing conductivity and hardness, as well as in improving the appearance of the water. This indicates a successful validation of the reactor cleaning process. With regard to the microbiological

study, the following figure shows the assessment of the internal reactor surfaces by means of swab sampling.



Figure 15. Measurement of reactor surfaces using swabs

The reactor surfaces were assessed at the following locations after each cleaning step:

- **Point 1:** Refers to the area of the mixing blades.
- **Point 2:** Refers to the reactor walls.
- **Point 3:** Refers to the bottom area of the reactor. A limitation of this area is that a small amount of water tends to remain, which may influence bacterial growth. This point is therefore prone to contamination.

A general aerobic count and specific microorganism counts (*Escherichia coli*, *Pseudomonas*, *Staphylococcus aureus*, *Aspergillus*, *Candida*, and *Burkholderia*) were performed on each washing water sample and on different internal reactor locations using swab sampling. The results obtained are presented below; blue color corresponds to washing water samples, while pink color corresponds to reactor surface samples collected using swabs.

Sample	Aerobic bacteria (≤ 1000 UFC/g)	Molds and yeasts (≤ 1000 UFC/g)	E.Coli	Pseudomonas	S.Aureus	Candida	Burkholderia	Compliance
Permeate water	<10	<10	Absent	Absent	Absent	Absent	Absent	COMPLIANT
Cleaning 1	<10	<10	Absent	Absent	Absent	Absent	Absent	COMPLIANT
Cleaning 2	<10	<10	Absent	Absent	Absent	Absent	Absent	COMPLIANT
Cleaning 3 (1)	<10	<10	Absent	Absent	Absent	Absent	Absent	COMPLIANT
Cleaning 3 (2)	10	<10	Absent	Absent	Absent	Absent	Present	NON-COMPLIANT
Cleaning 1 (1)	<10	<10	Absent	Absent	Absent	Absent	Absent	COMPLIANT
Cleaning 1 (2)	<10	<10	Absent	Absent	Absent	Absent	Absent	COMPLIANT
Cleaning 1 (3)	<10	<10	Absent	Absent	Absent	Absent	Absent	COMPLIANT
Cleaning 2 (1)	<10	<10	Absent	Absent	Absent	Absent	Absent	COMPLIANT
Cleaning 2 (2)	<10	<10	Absent	Absent	Absent	Absent	Absent	COMPLIANT
Cleaning 2 (3)	<10	<10	Absent	Absent	Absent	Absent	Absent	COMPLIANT
Cleaning 3 (1)	10	<10	Absent	Absent	Absent	Absent	Present	NON-COMPLIANT
Cleaning 3 (2)	<10	<10	Absent	Absent	Absent	Absent	Absent	COMPLIANT
Cleaning 3 (3)	<10	<10	Absent	Absent	Absent	Absent	Absent	COMPLIANT

According to the results obtained, during the third cleaning stage, corresponding to the water rinsing step, the presence of *Burkholderia* was detected in the second replicate. This same bacterium was also identified in swab samples taken from the mixing blade area after this cleaning step.

As the initial microbiological results did not comply with the established limits, the reactor cleaning validation process using treated wastewater for the first rinse was repeated. In this second trial, and as a corrective measure in response to the non-compliances detected with respect to the applicable



regulations, certain cleaning process parameters were modified, notably including an increase in agitation times, as well as adjustments to temperature and other operational conditions. These modifications were implemented in order to improve the effectiveness of the procedure and to promote a more complete removal of the microbiological load.

The following image shows the washing water samples collected after each stage of the reactor cleaning validation process carried out under these new operating conditions.

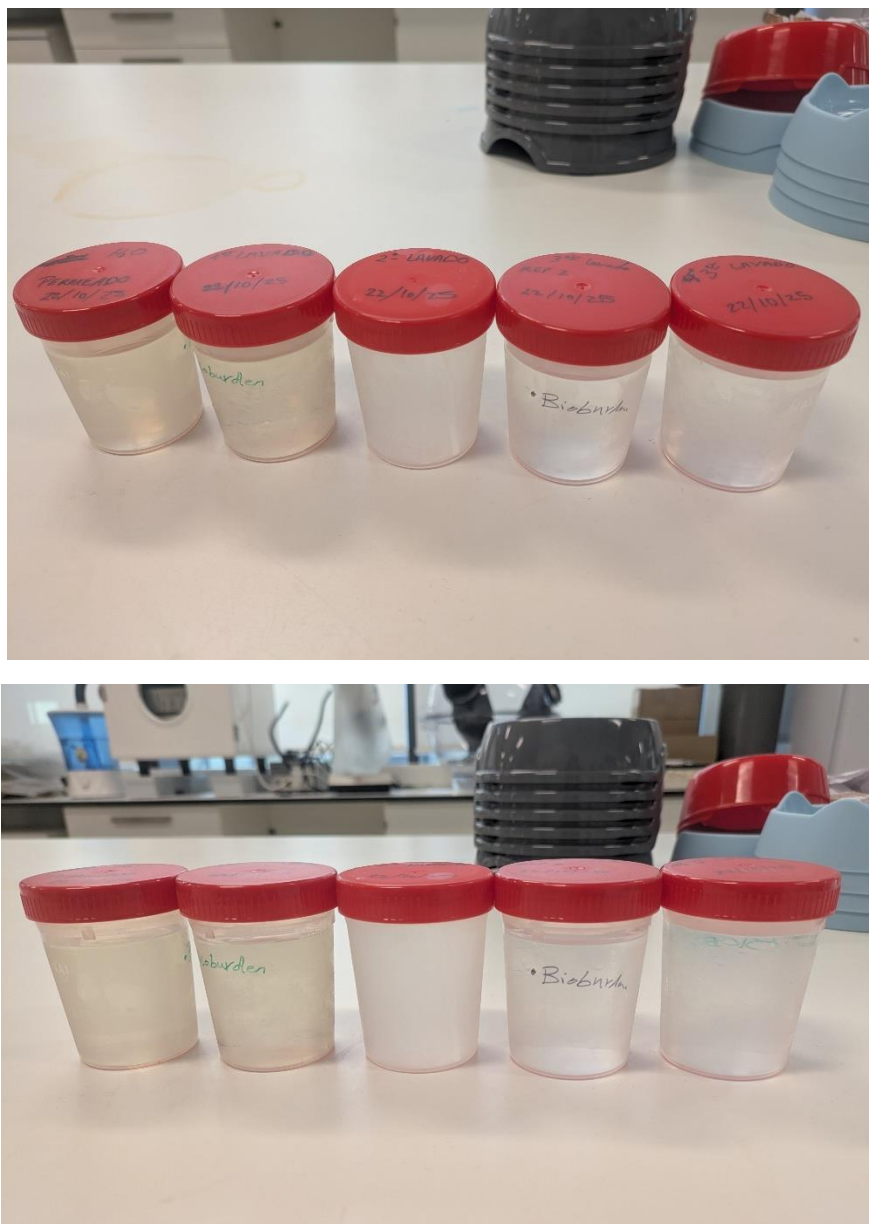


Figure 16. Photographs of the wash waters collected after each step in the cleaning validation of a cosmetic reactor using treated water from the Ecosite Spain pilot plant. From left to right: treated water, first wash water, second wash water, third wash water and third wash water (replicate 2).

The samples obtained were subjected to an evaluation of their microbiological properties and to the analysis of various physicochemical parameters, including pH, conductivity, and hardness, in order to verify the effectiveness of the cleaning process following the increase in agitation times.

Table 19. Physicochemical parameters of water samples after each washing step.

Sample	pH	Conductivity ($\mu\text{S/cm}$)	Hardness (mg/L)	Appearance
Permeate water	7,40	2133	295	Yellowish
Cleaning 1 (permeate water)	7,3	2120	298	Yellowish
Cleaning 2 (base–acid)	9,60	1460	< 2	Colorless
Cleaning 3 (rinse water)	8,1	12	< 2	Colorless
Cleaning 3 (rinse water) replicate	8,2	13	< 2	Colorless

The following image shows the swabs used for sampling the reactor surfaces during the cleaning validation process. These swabs were used for the microbiological evaluation of the surfaces after each stage of the procedure, with the aim of verifying proper contamination control and the effectiveness of the applied cleaning process.



Figure 17. Photograph of the swabs employed during the reactor cleaning validation process using treated wastewater.

The following table shows the values obtained after the cleaning process. Values highlighted in blue correspond to the washing water samples, while values highlighted in pink correspond to reactor surface samples collected using swabs.

Sample	Aerobic bacteria ($\leq 1000 \text{ UFC/g}$)	Molds and yeasts ($\leq 1000 \text{ UFC/g}$)	E.Coli	Pseudomonas	S.Aureus	Candida	Burkholderia	Compliance
Permeate water	180000	<10	Absent	Present	Absent	Absent	Absent	NON-COMPLIANT
Cleaning 1	150000	<10	Absent	Present	Absent	Absent	Absent	NON-COMPLIANT
Cleaning 2	<10	<10	Absent	Present	Absent	Absent	Absent	COMPLIANT
Cleaning 3 (1)	<10	<10	Absent	Absent	Absent	Absent	Absent	COMPLIANT
Cleaning 3 (2)	10	<10	Absent	Absent	Absent	Absent	Absent	COMPLIANT
Cleaning 1 (1)	<10	<10	Absent	Absent	Absent	Absent	Present	COMPLIANT
Cleaning 1 (2)	<10	<10	Absent	Absent	Absent	Absent	Absent	COMPLIANT
Cleaning 1 (3)	<10	<10	Absent	Present	Absent	Absent	Absent	COMPLIANT
Cleaning 2 (1)	<10	<10	Absent	Absent	Absent	Absent	Absent	COMPLIANT
Cleaning 2 (2)	<10	<10	Absent	Absent	Absent	Absent	Absent	COMPLIANT
Cleaning 2 (3)	<10	<10	Absent	Absent	Absent	Absent	Absent	COMPLIANT
Cleaning 3 (1)	10	<10	Absent	Absent	Absent	Absent	Absent	COMPLIANT
Cleaning 3 (2)	<10	<10	Absent	Absent	Absent	Absent	Absent	COMPLIANT
Cleaning 3 (3)	<10	<10	Absent	Absent	Absent	Absent	Absent	COMPLIANT

The results corresponding to the first rinse with permeate water (70% degreasing) show a very high microbiological load, with aerobic bacteria counts of 180,000 and 150,000 CFU/g in the permeate water and Cleaning 1 samples, respectively. In addition, the presence of *Pseudomonas* was detected in both samples. These results indicate that permeate water, when used as the first rinse, is not sufficient to effectively reduce the initial microbiological load of the reactor and may also act as a source of microbiological contamination. For this reason, these stages are considered non-compliant according to the established criteria.

After application of the alkaline treatment with 2% NaOH at 70 ° C, followed by rinsing and the acidic (biocidal) treatment at 3%, a drastic reduction in the microbiological load was observed. In the samples corresponding to Cleaning 2, aerobic bacteria counts as well as mould and yeast counts were below 10 CFU/g, and no presence of the indicator microorganisms analysed (*E. coli*, *S. aureus*, *Candida* and *Burkholderia*) was detected. Although *Pseudomonas* was still detected in the water at this stage, the overall results complied with the established microbiological limits, demonstrating the effectiveness of the chemical disinfection process in reducing contamination.

The results obtained after Cleaning 3 and the final rinse with distilled water showed consistently low microbiological values, with counts ≤ 10 CFU/g and a general absence of pathogenic and indicator microorganisms. Both water samples and swab analyses from the different areas of the reactor (mixing blades, walls, and bottom area) yielded compliant results, confirming that the complete cleaning process is effective following the application of the alkaline and acidic steps.

It is noteworthy that the bottom area of the reactor (Point 3), previously identified as a critical point due to water accumulation, did not show significant residual contamination after the complete cleaning process. This suggests that the increase in agitation and emulsification times, together with the applied temperature and chemical concentration conditions, was decisive in improving the effectiveness of soil removal and disinfection in areas most prone to bacterial growth.

Overall, the results demonstrate that although the initial rinse with permeate water does not ensure adequate microbiological reduction, the complete cleaning and disinfection process—when properly adjusted in terms of time and operating conditions—allows acceptable microbiological levels to be achieved in both the washing water and reactor surfaces, thereby validating the effectiveness of the applied procedure.

In light of the results obtained, which evidenced a high initial microbiological load associated with the use of treated wastewater during the preliminary rinse, it was decided to implement an additional control step by chlorinating the treated wastewater prior to its use. To this end, the free chlorine concentration was adjusted to 2 ppm, a value considered sufficient and widely used for the effective reduction of microbiological load in industrial water applications. Following the application of this measure, the initial rinse was repeated and the microbiological status of both the water and the reactor surfaces was evaluated.

The results obtained after chlorination were satisfactory, showing a significant reduction in the initial microbiological load. Microbiological control was carried out using the Ensure Touch system, employing the AquaSnap swab for water samples and the specific surface swab (UltraSnap Total) at critical reactor locations. In all cases, the results indicated the absence of microbiological contamination, confirming the effectiveness of chlorination of treated wastewater as a preventive measure to improve the microbiological safety of the cleaning process from the initial stage.



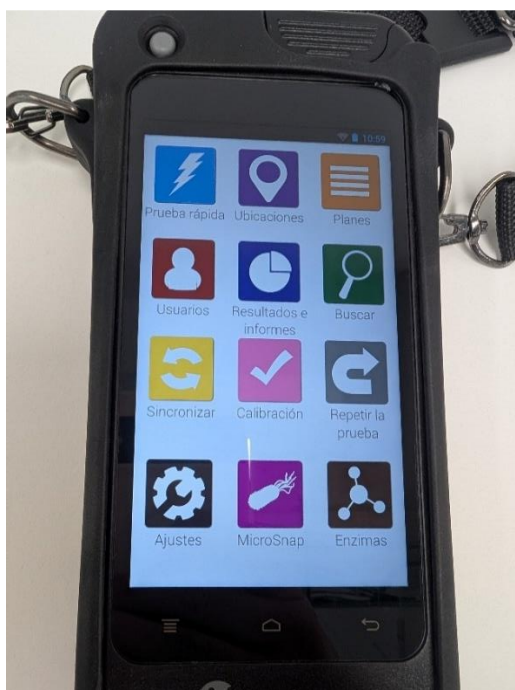


Figure 18. Ensure Touch system for the validation of cleaning of cosmetic reactors.

- **Reuse of Cosmetic Wastewater for Reactor Cleaning**

In addition to the trial carried out using treated wastewater from the Ecosite España pilot plant, an equivalent study was conducted using treated wastewater generated by the internal treatment process of the company Germaine, which actively collaborated in the development of this work. The objective of this trial was to evaluate the feasibility of reusing treated wastewater from the cosmetic production process itself for application in reactor cleaning and validation operations, in line with circular economy strategies and the efficient use of water resources.

The reactor cleaning validation procedure was performed under the same operating conditions previously described, maintaining the sampling points at the critical areas of the equipment (mixing blades, reactor walls, and bottom area), as well as the different stages of cleaning, disinfection, and rinsing. In this way, the comparability of the results obtained was ensured, allowing an objective evaluation of the microbiological performance of the treated wastewater from Germaine as an alternative for reuse in cleaning processes.

During the trial, washing water samples were collected after each stage of the process, together with surface samples taken by swabbing at the defined reactor locations. All samples were subjected to general and specific microbiological analyses, as well as to the determination of relevant physicochemical parameters, in order to assess the effectiveness of the cleaning process and the suitability of the treated wastewater from Germaine's treatment process for reuse in a cosmetic environment.

The following image shows the microbiological assessment carried out using the Ensure Touch system, employing the AquaSnap Total swab, which enables rapid detection of microbiological contamination through the measurement of ATP (adenosine triphosphate) present in the sample. This system is based on a bioluminescent reaction in which ATP reacts with a specific reagent, generating a light signal that is quantified by the device in RLU (Relative Light Units), which are indicative of the level of microbiological contamination. In the initial measurement performed on the



treated cosmetic wastewater from Germaine's treatment process, a value of 703 RLU was obtained, reflecting a high initial microbiological load.

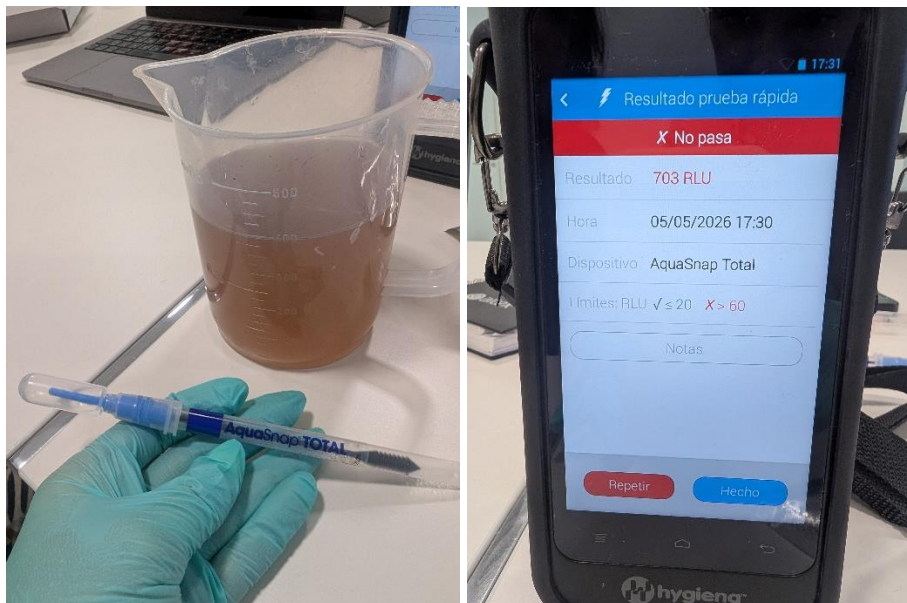


Figure 19. Validation of reactor cleaning using cosmetic wastewater with the Ensure Touch system.

However, after application of the complete reactor cleaning process, the measurement of the final washing water showed a value of 0 RLU, indicating the absence of detectable contamination and confirming the effectiveness of the cleaning procedure and the use of treated wastewater under the established conditions.

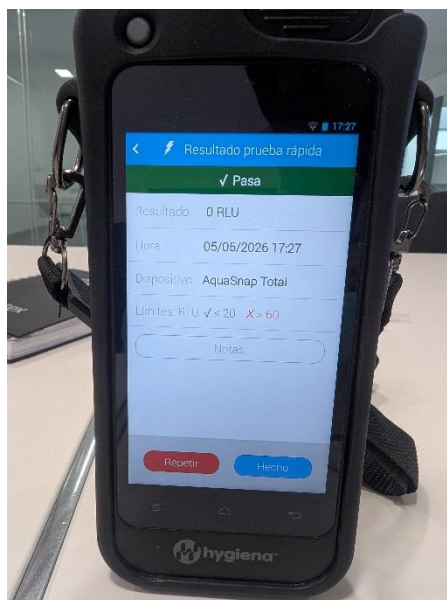


Figure 20. Validation of reactor cleaning using cosmetic wastewater with the Ensure Touch system.

The results obtained made it possible to validate the use of treated wastewater from Germaine's treatment process in the reactor cleaning procedure, provided that appropriate control and disinfection conditions are applied. In this regard, the collaboration with Germaine has highlighted



the high potential for reuse of treated wastewater within the cosmetic industry itself, contributing significantly to the reduction of potable water consumption and to the development of more sustainable processes.

In addition, the trials carried out showed that the use of treated cosmetic wastewater from Germaine presents certain operational advantages compared to treated wastewater from the pilot plant, as it starts from a lower initial microbiological load. This condition facilitates validation of the cleaning process effectiveness, allowing the established microbiological criteria to be met with less stringent requirements for parameters such as agitation time, temperature, or process intensity. Nevertheless, both types of treated wastewater proved to be suitable for reuse in reactor cleaning, provided that appropriate control measures are implemented.

