This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 730285

Project Partners:

1. AQUALIA
2. DESAH
3. SLU
4. LEAF BV
5. LEITAT
6. NSVA
7. USC
8. WE&B
9. WU
10. ZFV
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13. CEIP
14. 4F
15. ASB

RECOVERY AND UTILIZATION OF NUTRIENTS 4 LOW IMPACT FERTILIZER

H2020-CIRC-2016TwoStage

Collaborative project

Start date of the project: 01/06/2017
Duration 48 months

D4.3
Assessment of a quality and safety of water reuse for industrial and agricultural application

WP	4	End-users perspective: quality and safety requirements

Dissemination level[1]	PU
Nature[2]	R

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[1] Dissemination level: **PU** = Public, **PP** = Restricted to other programme participants (including the JU), **RE** = Restricted to a group specified by the consortium (including the JU), **CO** = Confidential, only for members of the consortium (including the JU)
[2] Nature of the deliverable: **R** = Report, **P** = Prototype, **D** = Demonstrator, **O** = Other

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V.1	15/11/2021	NMP	AQUA	Nicolas.morales.pereira@fcc.es	Modification
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V.2	14/'2/2022	NMP	AQUA	Nicolas.morales.pereira@fcc.es	Final version following evaluation

[3] Creation, modification, final version for evaluation, revised version following evaluation, final

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List of acronyms and abbreviations

AnMBR	Anaerobic MBR
BOD	Biological Oxygen Demand
W	Blackwater
COD	Chemical Oxygen Demand
dsRNA	Double-Stranded RNA
EC	Electrical Conductivity
G	Genogroups
GC-MC	Gas Chromatography Mass Spectrometry
GW	Greywater
IC50	Inhibitory Concentration 50
LC50	Lethal Concentration 50
MBR	Aerobic Membrane Bioreactor
MP	Micropollutants
NoV	Norovirus
OMP	Organic Micropollutants
PE	Person Equivalents
RNA	Ribonucleic Acid
SaV	Sapovirus
ssRNA	Single-Stranded RNA
TSS	Total Suspended Solids
UV	Ultraviolet Light
WWTP	Wastewater Treatment Plant

Deliverable abstract

The present report represents the delivery of *Task T4.3. Assessment of a quality and safety of water reuse for industrial and agricultural applications* of Run4Life project, and it is part of the *WP4 End-users perspective: quality and safety requirements*.

The task concerns evaluating the applicability of the reclaimed water in agriculture as nutrient irrigation water, and possible uses in industry. The present report describes the legislative barriers and regulatory framework for water reuse and the activities and tests carried out in this regard at the demo sites.

The reuse of reclaimed water in agriculture was evaluated by using AnMBR effluent from treated black water from Vigo to irrigate ray grass and basil in pot experiments conducted in two countries covering different climatic conditions (temperature, humidity) as well as soil types. Even if yield differed between experiments and crops and soil type the overall trend was the same: supplying nutrients by fertigation gave an increased yield compared to irrigation with water, the yield was however lower compared to pots receiving similar, or even lower, dose of mineral fertiliser as an initial application. The results indicate that the fertigation (which was based on the plants water need) as the sole source of nutrients resulted in a nutrient application that did not fully match the growth rate of the crop. Thus, it would be beneficial to combine fertigation with some inital fertilisation and not having the fertigation water as the sole source of nutrients since the inital low water requirements of the plants may result in initial low doses on fertiliser. On the other side, if irrigation need is large the nutrients applied with the fertigation may result in a large total dose of nutrients that can exceed a normal fertiliser application. Any application of other fertiliser thus need to consider the nutrients applied with the fertigation.

The macro- and micro-nutrient contents, the amount of heavy metals and micropollutants in plants biomass will be analysed with standard methods (ISO 11885) at WU. These analytical data (composition of plant biomass) will be used in WP5 to estimate risks for human health and the environment. In parallel to soil tests, LEITAT will be in charge of performing toxicity tests of the irrigation water by means of standard tests (US EPA 850.4200: Seed Germination/Root Elongation Toxicity Test and OECD Test No. 207: Earthworm, Acute Toxicity Tests).

The demosite in Ghent will deliver the recycled water as process water at an adjacent chemical plant. The water will be tested on physico-chemical parameters to allow subsequent treatment in a reverse osmosis system. The presence of organic micropollutants will be evaluated by USC by means of Gas Chromatography Mass Spectrometry (GC-MC) and/or other chromatographic analysis. Moreover, degree of maturity and pathogens content (*E. coli*, *Salmonella* spp., *Legionella* spp., helminths and viruses) will be determined.

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This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 730285.

1. Introduction

The present report represents the delivery of *Task T4.3. Assessment of the quality and safety of water reuse for industrial and agricultural application* project.

Water is a limited resource in the EU, with one third of the EU territory experiencing water stress. The growing needs of populations and climate change will make the availability of water in sufficient quantity and quality even more of a challenge in Europe in the future. Water over-abstraction, in particular for agricultural irrigation but also for industrial use and urban development is one of the main threats to the EU water environment, while availability of water of appropriate quality is a critical condition to growth in water-dependent economic sectors and society in general.

In a circular economy, water reuse plays a key role, bringing significant environmental, social, and economic benefits. There is high potential for increased water reuse, but awareness of the benefit of this technology is low, and Europe lacks an adequate supportive framework for water reuse.

This report evaluates the applicability of the reclaimed water in agriculture as irrigation water with nutrients, and possible uses in industry or in the buildings, according to reclaimed water quality regulation (EU)2020/741 of 25 May 2020 on minimum requirements for water reuse.

The presence of emerging pollutants in aquatic environments has already been considered by the Water Framework Directive, which establishes a "Watch List" and a priority list of substances. In this sense, some Organic Micropollutants (OMP´s) have to be monitored, while the concentration of priority pollutants is limited. On contrary, the European regulation on sewage sludge use in agriculture (Directive 86/278/EEC) disregards the presence of most OMPs.

Overall, the present report evaluates the results obtained in terms of quality and safety reclaimed water in agricultural and industrial application:

- Overall description of demo sites with water reclamation: Vigo demo site (Spain) and Ghent demo Site (Belgium).
- Results of quality and safety of reclaimed water.

2. Overall description of the two demo sites

The present report represents a summarizes progress in terms of quality and safety reclaimed water, evaluating the applicability of the re-claimed water in agriculture as irrigation water with nutrients and as water reuse for toilet flushing in Vigo demo site, and possible uses in industry in Ghent demo site.

2.1 Vigo demo site, Spain

Vigo demo site is located in the business center "Centro de Negocios Porto do Molle", in Nigrán (Pontevedra) close to the city of Vigo in Spain. Prior to the Run4Life project the business center (Hosting approximately 200-250 people during working hours) was already equipped with segregated grey and black water collection in all restrooms (Figure 1 and Figure 2). These streams were partially treated in a local WWTP located in the underground parking lot.

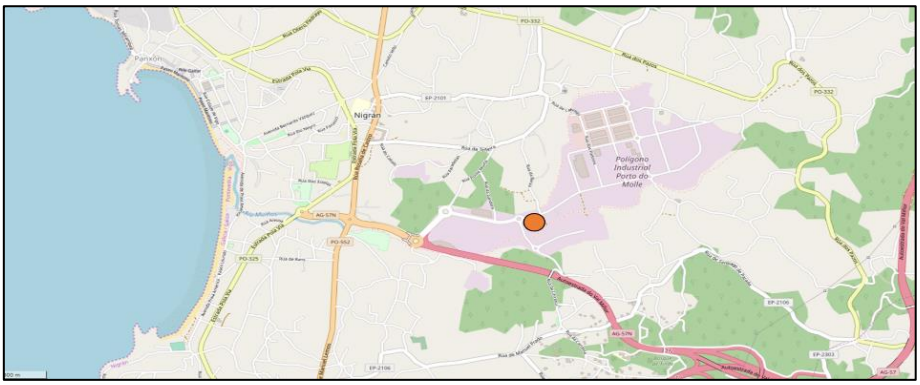

Figure 1: Location of the demo site at the building Centro de Negocios Porto do Molle in Porto do Molle Business park, close to the city of Vigo. Image from openstreetmap.org.

Figure 2: Centro de Negocios Porto do Molle building in Porto do Molle Business Park. Photo: Zona Franca

Grey water was collected and treated in an aerobic MBR and finally disinfected dosing hypochlorite, stored and used to refill toilet cisterns, while blackwater was collected in a cesspit. A blackwater treatment line was installed, consisting of a 2 m^3 buffer tank, a 2.4 m^3 anaerobic MBR (AnMBR) with 1 m^3 external membrane tank. In order to monitor biogas produced in the AnMBR a biogas piping system and methane meter were installed. As a tertiary treatment of the produced AnMBR effluent as well as disinfection of treated greywater, UV-LED treatment was installed in order to obtained quality and safety reclaimed water to use in agriculture. Complete treatment scheme is shown in Figure 3.

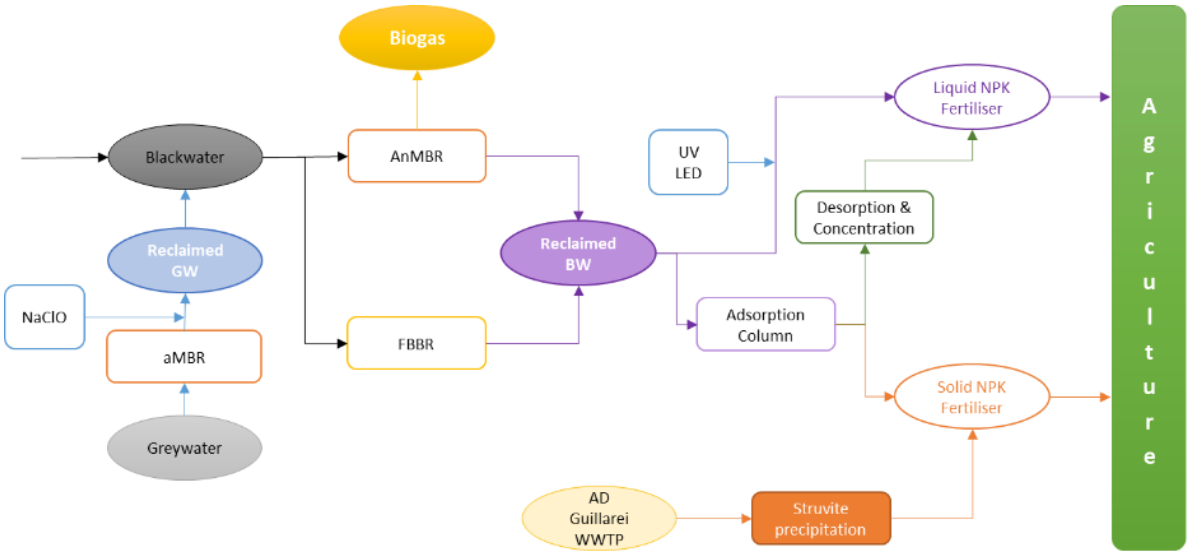

Figure 3 : Run4LifeTreatment scheme in Vigo demo site

2.2 Ghent demo site, Belgium

In Ghent, Belgium, an old harbour area in Dampoort is rebuilt into a new residential district (

Figure *4*), including source separation wastewater system for blackwater, greywater and food waste. The new district is called The Nieuwe Dokken and will be completed in 3 phases, the central field, the north field and the south field (Figure 5). The central field contains a school, a sports complex, a day care, a building with 90 apartments and an underground local wastewater treatment plant. The central field is finished (Figure 6). The north field will contain approximately 100 housing units and will be finished in 2020 – 2022. The south field contains approximately 200 housing units and will be finished in 2022 – 2024. The site will, when completely finalized in 2024, accommodate more than 400 housing units, office spaces, shops and public buildings.

The project is operated by a cooperative (DuCoop) who will manage wastewater and food waste treatment. The vacuum sewage system will collect blackwater and kitchen waste from 1250 person equivalents (PE) by 2024 and it is expected that all of the central field will be using the system by the end of Run4Life. Grey water will be treated for industrial reuse at a local neighbouring soap factory (Christeyns), but this is not included in the Run4Life project.

Figure 4: Location of the demo site of Ghent. Image from openstreetmap.org

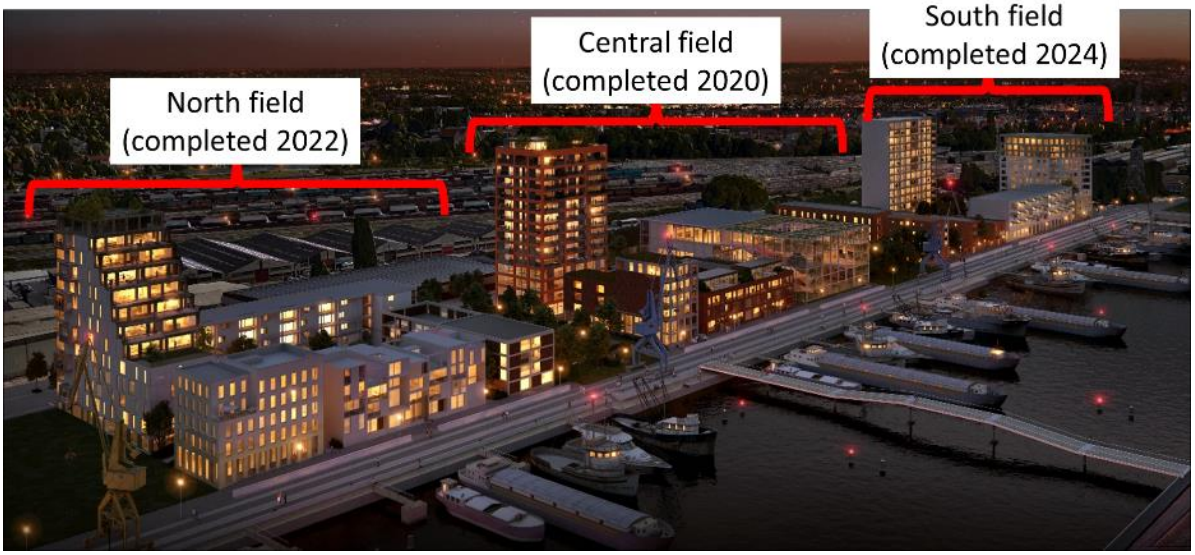

Figure 5: Visualisation of the Nieuwe Dokken district, including timelines for completion of the building phases

The suggested treatment scheme from the Run4Life proposal is shown in Figure 7. As made clear, the demo site will have blackwater collection with a vacuum sewer system (developed by Roediger) and food waste collection from central collective use food waste disposers. The combined wastewater stream is anaerobically digested in a UASB digester. Phosphorus is recovered from the decanted effluent after which the effluent wastewater is lead to lead to further polishing, including residual nitrogen, phosphorus and carbon removal processes (in the greywater treatment line).

The demosite in Ghent will deliver the recycled water as process water at an adjacent chemical plant. The water will be tested on physico-chemical parameters to allow subsequent treatment in a reverse osmosis system.

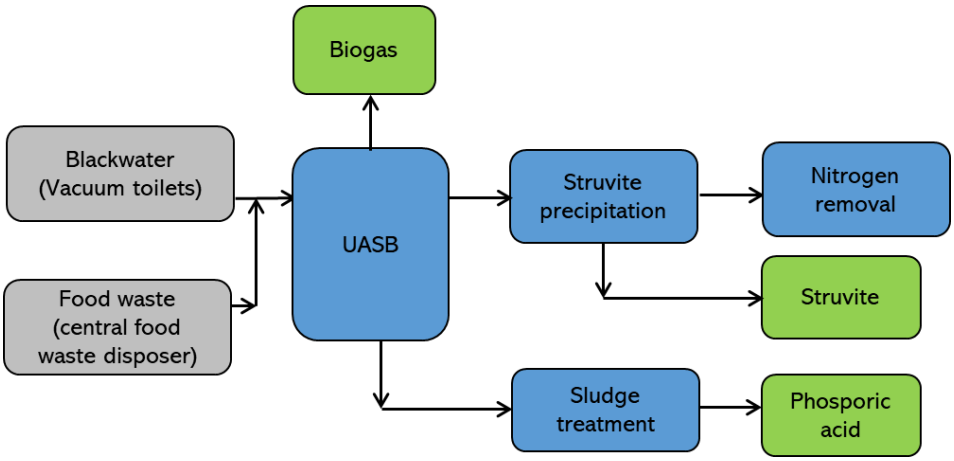

Figure 6: General treatment scheme of the Ghent demo site for source separated blackwater and food waste

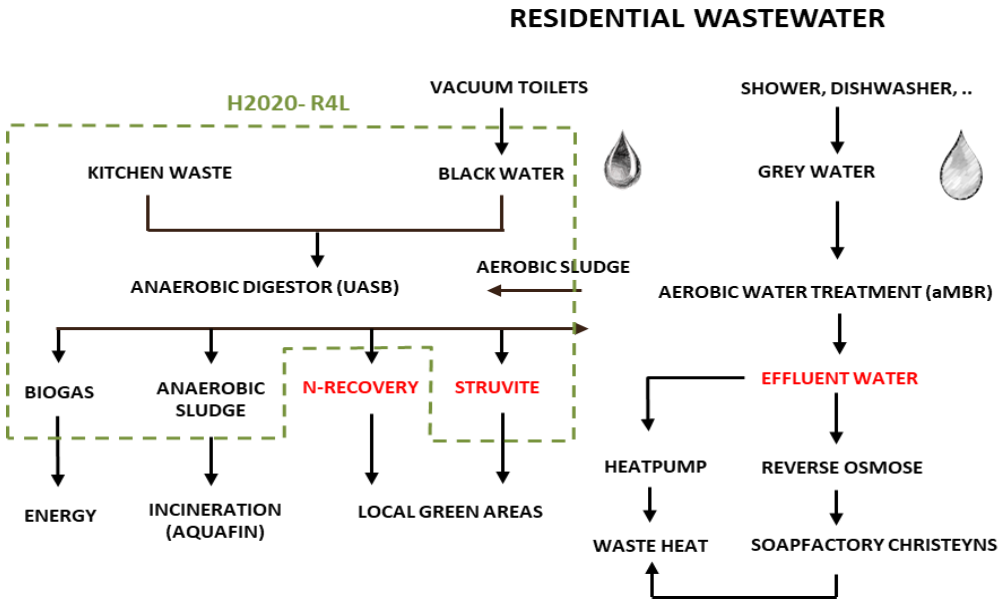

Figure 7: General treatment scheme of the Ghent demo site (Nieuwe Dokken), including greywater treatment and reuse, and heat recovery..

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3. Application in Vigo demo site

To evaluate the applicability of the re-claimed water, micropollutants, viruses and pathogens were sampled in Vigo demosite. The main objective is to verify that grey water is totally safe for use in the filling of the WC flush, while the AnMBR effluent complies with the strictest limits set for quality of water reuse framework.

3.1 Grey water reuse.

All the grey water collected was treated in an aerobic membrane bioreactor (MBR) with a maximum capacity of 1.5 m^3/d and is equipped with 6.25 m^2 of membrane, subsequently grey water was disinfected dosing hypochlorite, stored and used to refill toilet cisterns.

Inlet grey water characteristics (Table 1) are relatively variable with average values around 137 mg COD/L, 1.5 mg P/L and 24 mg N/L. The average volume of grey water treated and disinfected is 0.6 m^3/d, used to refill toilet cisterns in conventional gravity toilets (3-4.5 L/flush).

Disinfected system was based on a pulse counter that allows the addiction of 4 pulses/L of sodium hypochlorite. This dosage is carried out according to the permeate extraction flow of the membrane. The aim is for treated water ready to refill cisterns to contain an active chlorine concentration of 1 mg/L.

Table 1: Grey water characteristics in Porto do Molle bulding

Grey water characteristics at Porto do Molle	
Number of users	200-250 people during working hours
Treated maximum amount of grey water [m^3/d]	0.6
Incoming black water	
t COD (mg/L)	137± 71
TP (mg P/L)	1.5
TN (mg/L)	24
MBR-treated* Grey *water	
t COD (mg/L)	5.7
TP (mg P/L)	1.3
TN (mg/L)	11.1

3.2 Blackwater reuse.

The system is composed by a buffer tank with a capacity to store around 2 m^3 of black water. Black water stream is treated in an anaerobic membrane reactor (AnMBR), with a total volume of 2.8 m^3 and a membrane surface unit of 6.25 m^2 with a nominal pore size of 35 nm, maximum 0.1 µm.

The treatment at Porto do Molle has been operational within the Run4Life concept since March 2018 when the new AnMBR system was started up. Table 2 shows the concentration values in the demosite.

As tertiary treatment of the produced AnMBR effluent as well as disinfection of treated greywater, UV-LED treatment (Ultraviolet light in C range produced by Light Emission Diodes) was installed in the beginning of 2020 with the objective of performed disinfection tests of AnMBR permeate.

Table 2: Black water characteristics at PdM

Black water characteristics at Porto do Molle (Vigo)				
Flush volume [m^3]	3-4.5 L (conventional flush)			
Number of users	200-250 people during working hours			
Treated maximum amount of black water [m^3/d]	1.5			
Blackwater composition				
year	***2018***	***2019***	***2020***	***2021***
pH	7.5 ± 0.2	7.1 ± 0.2	7.2 ± 0.2	7.2 ± 0.3
Alkalinity (mg $CaCO_3$/L)	166 ± 39	98 ± 29	81 ± 12	81 ± 33
t COD (mg/L)	1571 ± 973	1288 ± 778	1179 ± 591	695 ± 428
TN (mg/L)	132 ± 37	89 ± 36	67 ± 18	72 ± 45
N-NH_4^+ (mg N/L)	22 ± 7	16 ± 9	12 ± 3	11 ± 5
TP (mg P/L)	1571 ± 973	1288 ± 778	1179 ± 591	695 ± 428

Permeate AnMBR composition				
year	***2018***	***2019***	***2020***	***2021***
COD_T (mg/L)	99 ± 48	75 ± 27	59 ± 14	57 ± 24
TP (mg/L)	19 ± 5.5	13 ± 3.5	10 ± 2	10 ± 4
TN (mg/L)	196 ± 78	187 ± 96	104 ± 34	113 ± 52
N-NH4 (mg/L)	145 ± 44	102 ± 36	69 ± 18	85 ± 38

3.2.1 Micropollutants

With the increasing technological advancements related to the production of desirable products, thousands of anthropogenic chemicals end up being discharged in the water resources. Commonly, these chemicals are referred to as micropollutants and are of paramount concern as their exposure may pose a significant risk to the aquatic ecosystem and human health due to their prevalence in the environment.

According to European Environment Agency, a micropollutant is a pollutant which exist in very small traces in water. The removal of anthropogenic micropollutants (MPs) emitting from domestic, industrial, agricultural, and urban sources are one of today's major global challenges (Alvarino et al., 2018b). Therefore, these micropollutants (MPs) have been the subject of study for many years due to their severe biological impacts (Aschermann et al., 2018; Batel et al., 2020; Gautam and Anbumani, 2020). The quantities of organic micropollutants such as contraceptive medicines, aromatic hydrocarbons, antibiotics, personal care products and pesticides are increasing day by day and reaching to the alarming level (Mailler et al., 2016; Meza et al., 2020). These accumulate in plants and animals then reach to humans through the food chain.

The list of emerging micropollutants includes a wide variety of everyday products with both industrial and domestic applications. Some of the most relevant are:

- Drugs (anti-inflammatories, analgesics, antibiotics, psychopharmaceuticals, proton pump inhibitors, lipid regulators, hormones)
- Personal care and hygiene products (sunscreens, disinfectants and fragrances),
- Microplastics, flame retardants, industrial additives, nanoparticles, drugs of abuse, etc.

These pollutants are, in general, at low concentrations (in the range of ng /L), but due to the increase in demand and consumption, coupled with the inability of treatment plants to completely eliminate them, they are continuously introduced into the environment and do not need to be persistent to cause negative effects

The MPs analysed in grey water streams in the project are:

- **CELESTOLIDE, TONALIE** and **GALAXOLIDE:** Are synthetic polycyclic fragrance.
- **CARBAMAZEPINE, DIAZEPAM, CITALOPRAM and FLUOXETINE:** Are included in the type of drugs called anticonvulsants, antidepressants, sedative treatments, and anxiolytic.
- **IBUPROFEN, NAXOPREN** y **DICLOFENAC:** They are nonsteroidal anti-inflammatory drugs.
- **4 OCTILFENOL** and **4 NONIFENOL:** They are common ingredients in detergents and cleaning products. They have the function of endocrine disruptors.
- **TRICLOSAN**: Antiseptic used in hospital products (hand washing and soaps) and consumer products (deodorants and toothpastes).
- **ESTRONE** and **ESTRADIOL:** Are natural estrogens secreted. They have the function of endocrine disruptors
- **ETILNYLESTRADIOL:** A Synthetic steroidal estrogen, derived from estradiol and also have functions of endocrine disruptors.
- **SULFAMETOXAZOLE, TRIMETHOPRIM, ERITROMICINE and ROXITROMYCINE:** antibiotic drugs for urinary and respiratory infections
- **BISPHENOL -A:** Chemical substance used in the synthesis of plastics, resins and have the function of endocrine disruptors

The methodology used for Micropollutants analysis was:

- Celestolide, Galaxolide and Tonalide, they are analysed by gas chromatography coupled to mass spectrometry.

- Carbamazepine, Diazepam, Eritromicine, Fluoxetina, Roxitromycine, Sulfametoxazole, Trimethoprim, Estrone, Estradiol, Etinylestradiol, Ibuprofen, Naproxen, Diclofenac, 4-Octilfenol, 4-Nonilfenol, Bisphenol A and Triclosan; they are analysed by liquid chromatography coupled to mass spectrometry.

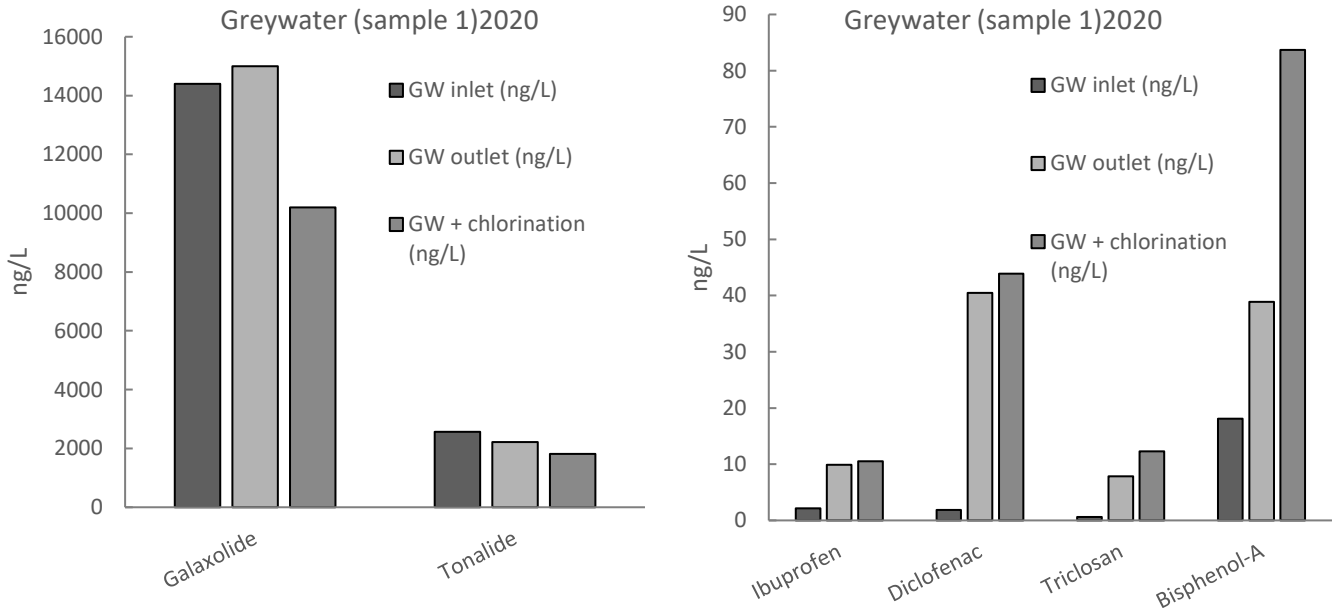
Grey water:

Grey water stream in Vigo demo site only include water from hand basin because plant is located into a Business Centre in which users only use the facilities during the 6-8 hours of their working day.

In 2020, two samplings (sample 1 and sample 2) were taken from the following process streams:

- Greywater inlet (GW inlet): which corresponds to the water generated by the handwashing
- Greywater outlet (GW outlet): is treated water after filtered process
- Greywater after chlorination (GW outlet +chlorination): is water stream after disinfected treatment

Only the MP´s detected with concentration greater than 1 ng/L are represented in the figures, since below that value it is impossible to make an accurate determination. In none of the cases we can refer to a removal percentage but have to refer to trends in removal concentration in the different samples carried out. Below shows graphs corresponding to 2020 grey water samples (Figure 8).



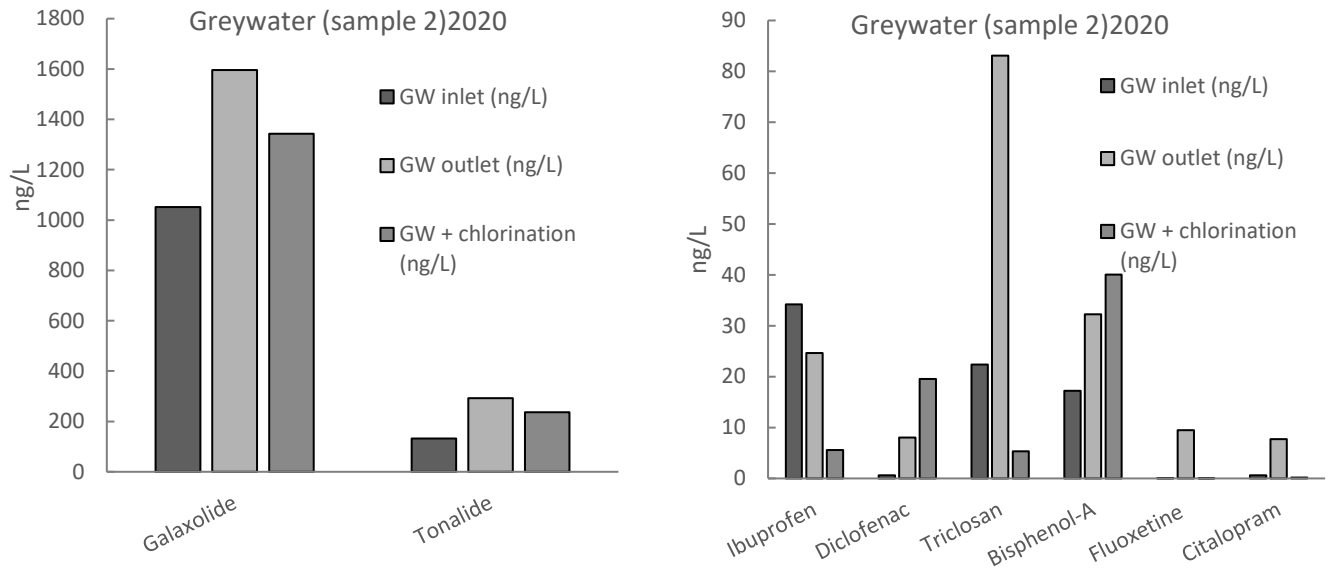
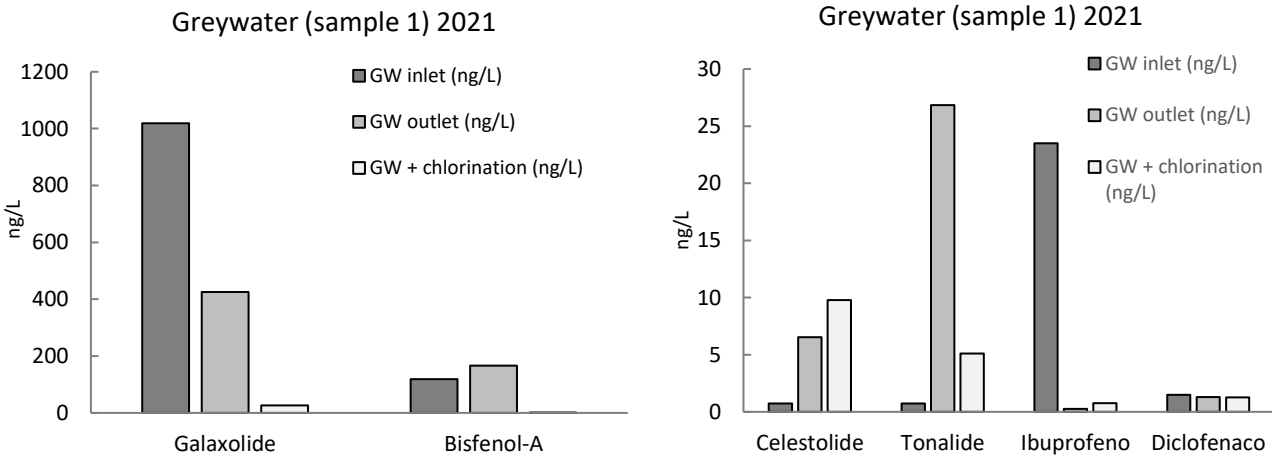


Figure 8: Grey water samples in 2020.

From the figures shown it can be concluded that trends are not comparable in two samplings. While in the first one exists a tendency to reduce polycyclic fragrance compounds, the second sample shows after passing through the membrane a slight reduction trend in Ibuprofen, Triclosan and Citalopram concentration.

It is decided to carry out a second sampling considering the hydraulic retention times in the tanks in order to obtain more conclusive results. In 2021 four sampling was carried out. Grey water influent sample was caught one day before grey water effluent and Grey water + Chlorination samples (*Figure 9* and *Figure 10*)



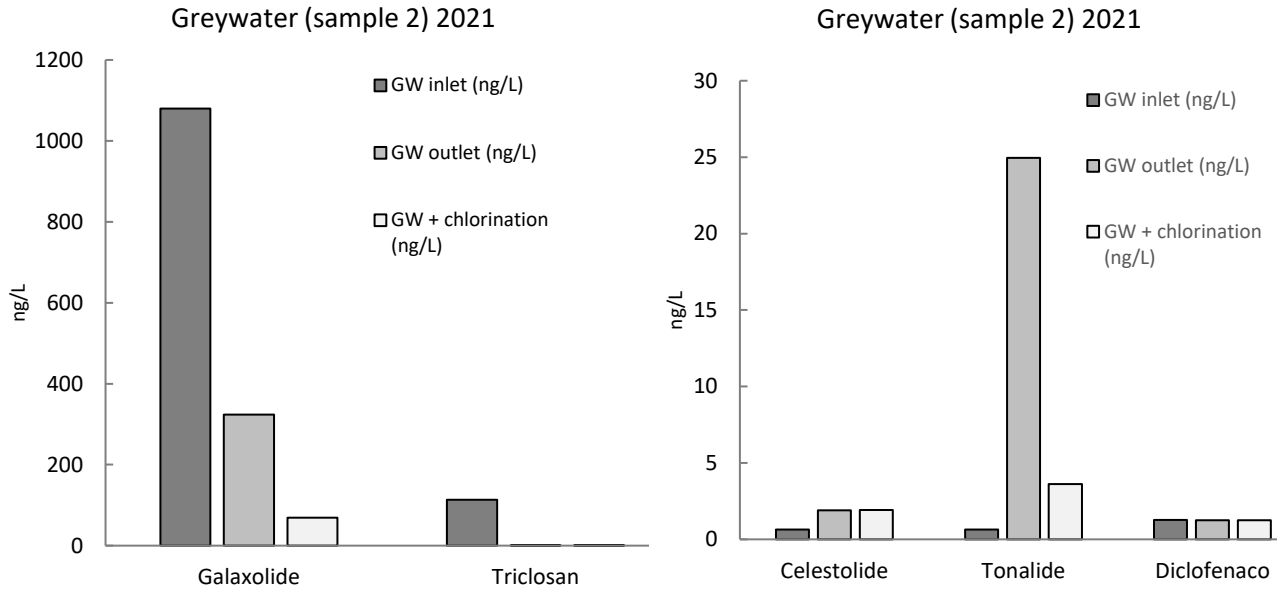
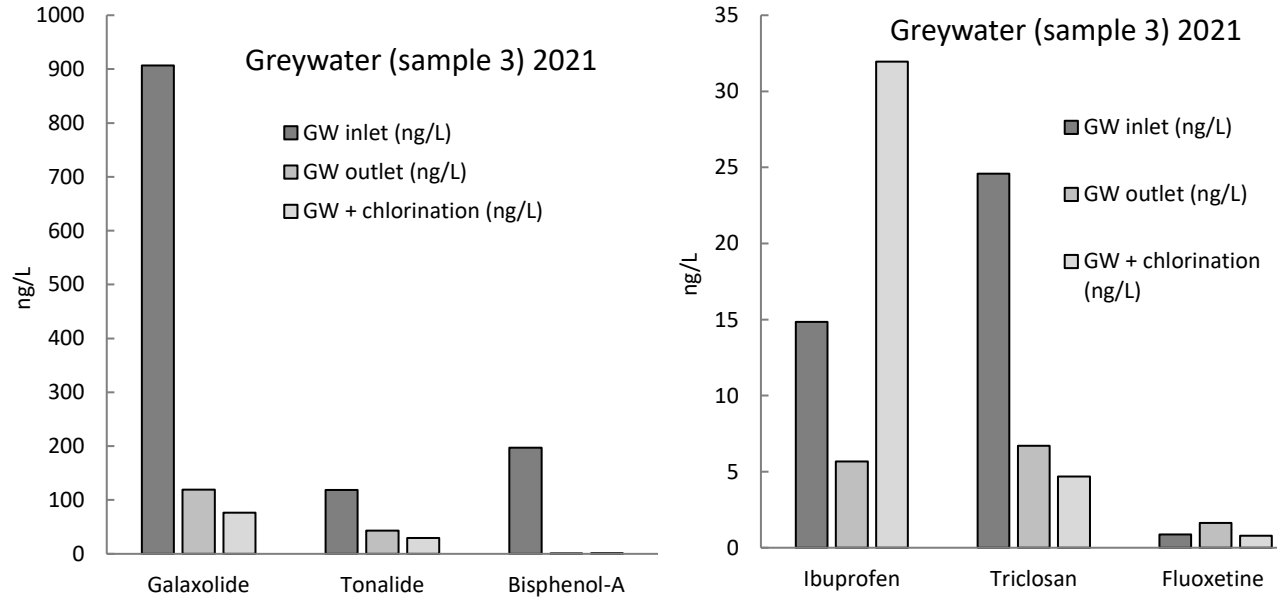


Figure 9: Grey water samples in 2021



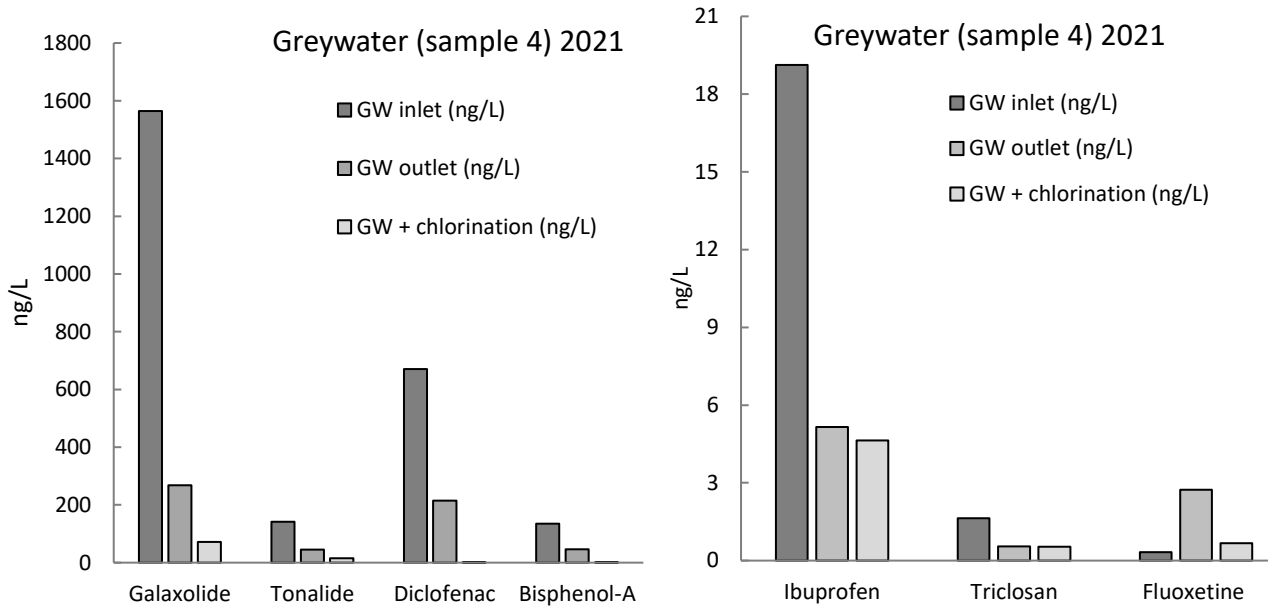


Figure 10: Grey water samples in 2021

From the sampling carried out in 2021, it can be observed a reduction of certain compounds due to the correct membrane performance and disinfection treatment effectiveness. Specifically, in Galaxolide and Tonaline (polycyclic fragrances), Triclosan and Bisphenol-A. In addition, an Ibuprofen removal trends exists in 2 of these 4 samplings.

Black water:

Despite of exclusively collecting the black water generated in the building itself, a seasonal variability associated with the presence of rainwater in the network can be observed. To this fact we must add that the number of people working in the building has decreased as a result of the Covid-19 pandemic and the consecutive promotion of working from home during most of 2020 and 2021.

In 2020, two samplings (sample 1 and sample 2) were taken from the following process streams:

- Blackwater inlet (BW inlet): which corresponds to the blackwater collected in the cesspit.
- Blackwater outlet (BW outlet): is the treated water after AnMBR process.
- Blackwater after disinfection (BW outlet + U.V): is water stream after disinfected treatment.

Analogous to explained above in the grey water, only the MPs detected with concentration greater than 1 ng/L are represented, since below that value it is impossible to make an accurate determination. In none of the cases we can refer to a removal percentage but have to refer to trends in concentration removal in the different samples.

Graphs corresponding to 2020 samples are shown below (*Figure 11*).

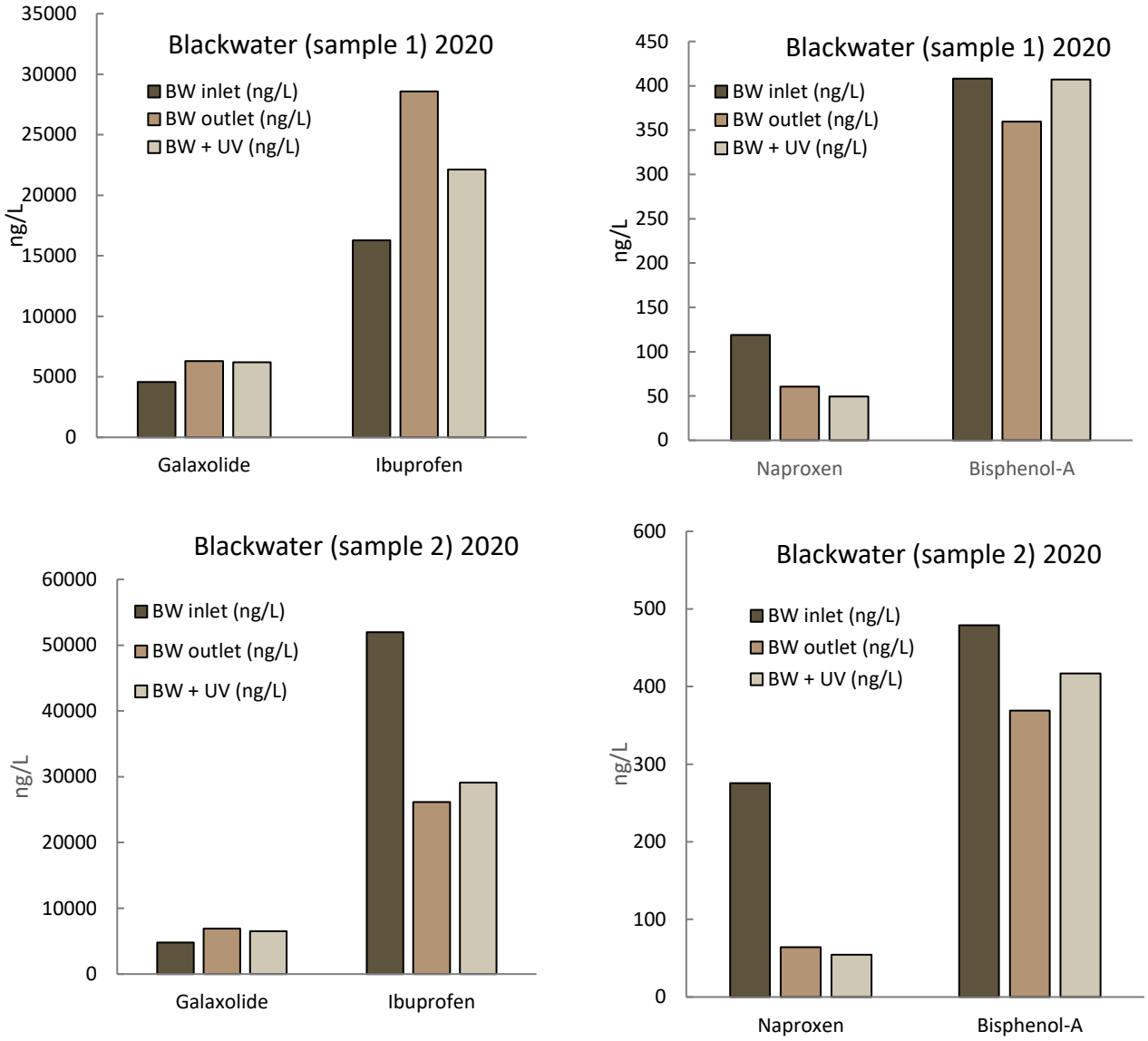
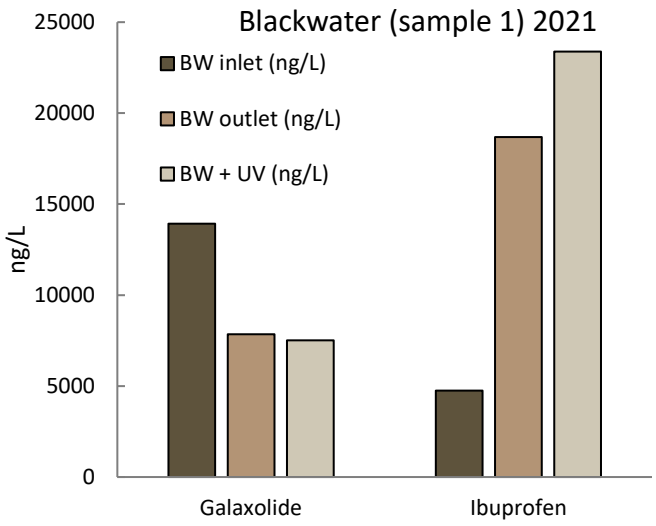


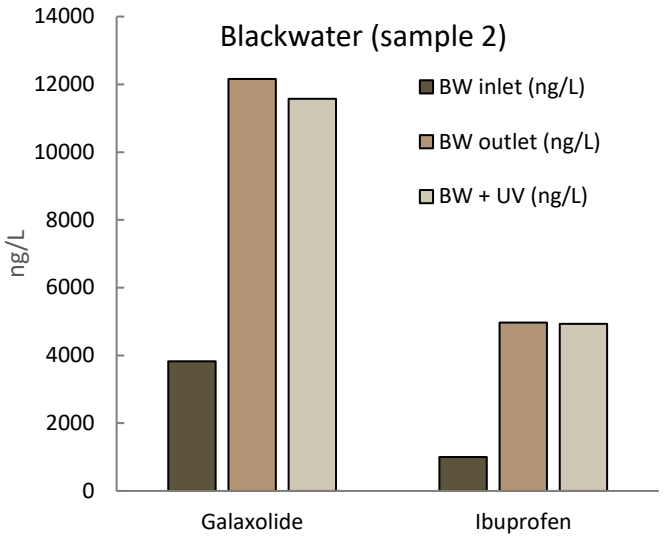
Figure 11: Black water samples in 2020

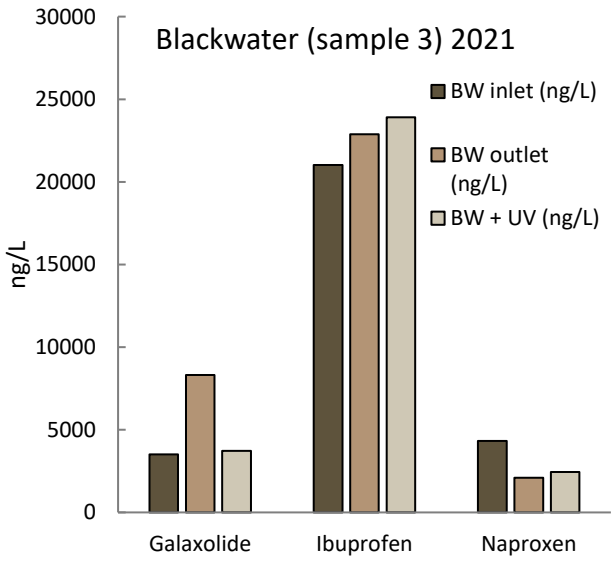
The results of both samples are very similar, Ibuprofen and Naxoprene (from the non-steroidal anti-inflammatory drugs family) as well as Galaxolide and Bisphenol-A are present in both.

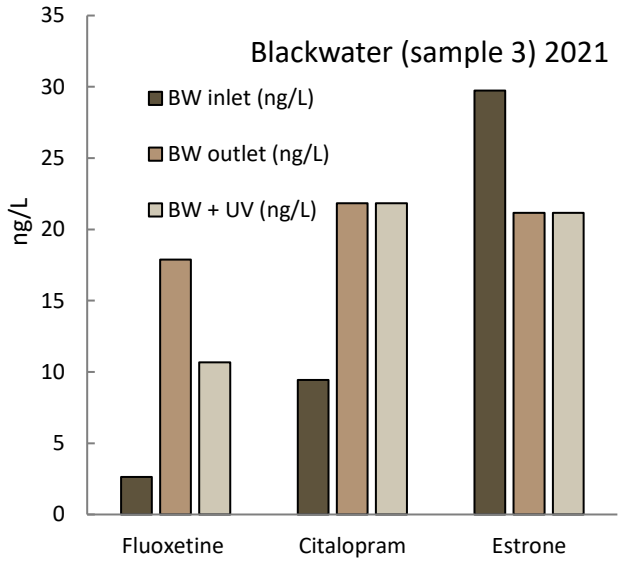
In some cases, higher concentrations of micropollutants can be observed after passing through the membrane. These data are not consistent with the proper membrane performance, and can be caused by accumulation of the micropollutants in the reactor, effect of the retention time, etc.

In order to obtain more conclusive results, in 2021 four sampling campaigns were carried out considering the hydraulic retention time in tanks. Raw blackwater samples was caught one day before effluent and U.V disinfection samples (*Figure 12*).


Blackwater (sample 1) 2021
BW inlet (ng/L)
BW outlet (ng/L)
BW + UV (ng/L)
ng/L
25000
20000
15000
10000
5000
0
Galaxolide
Ibuprofen


Blackwater (sample 2)
BW inlet (ng/L)
BW outlet (ng/L)
BW + UV (ng/L)
ng/L
14000
12000
10000
8000
6000
4000
2000
0
Galaxolide
Ibuprofen


Blackwater (sample 3) 2021
BW inlet (ng/L)
BW outlet (ng/L)
BW + UV (ng/L)
ng/L
30000
25000
20000
15000
10000
5000
0
Galaxolide
Ibuprofen
Naproxen


Blackwater (sample 3) 2021
BW inlet (ng/L)
BW outlet (ng/L)
BW + UV (ng/L)
ng/L
35
30
25
20
15
10
5
0
Fluoxetine
Citalopram
Estrone

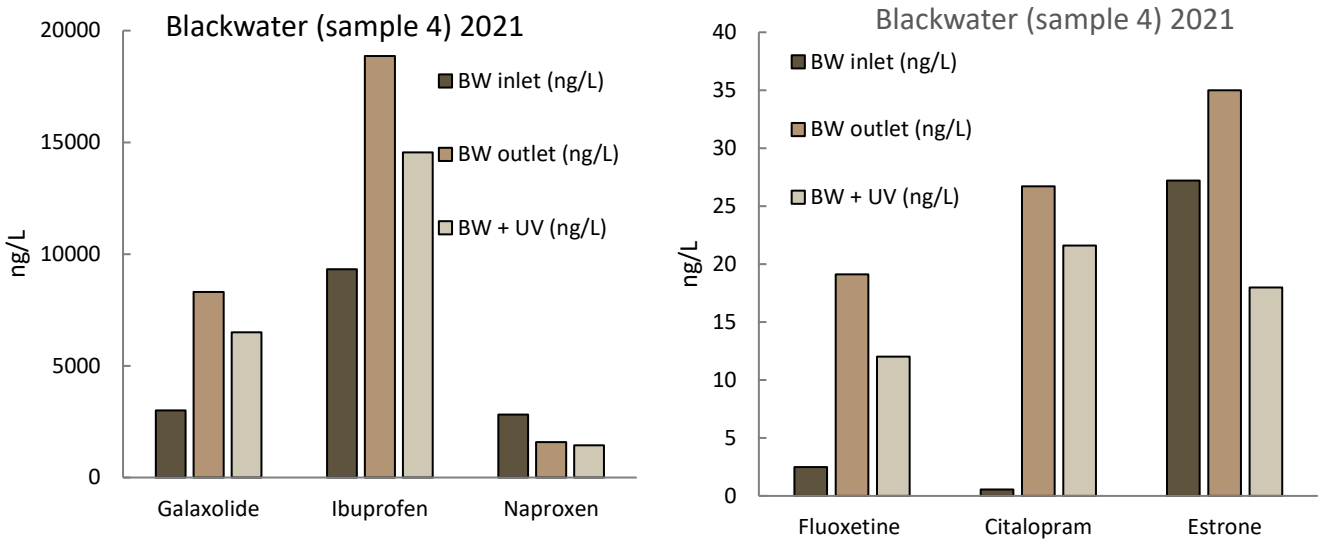


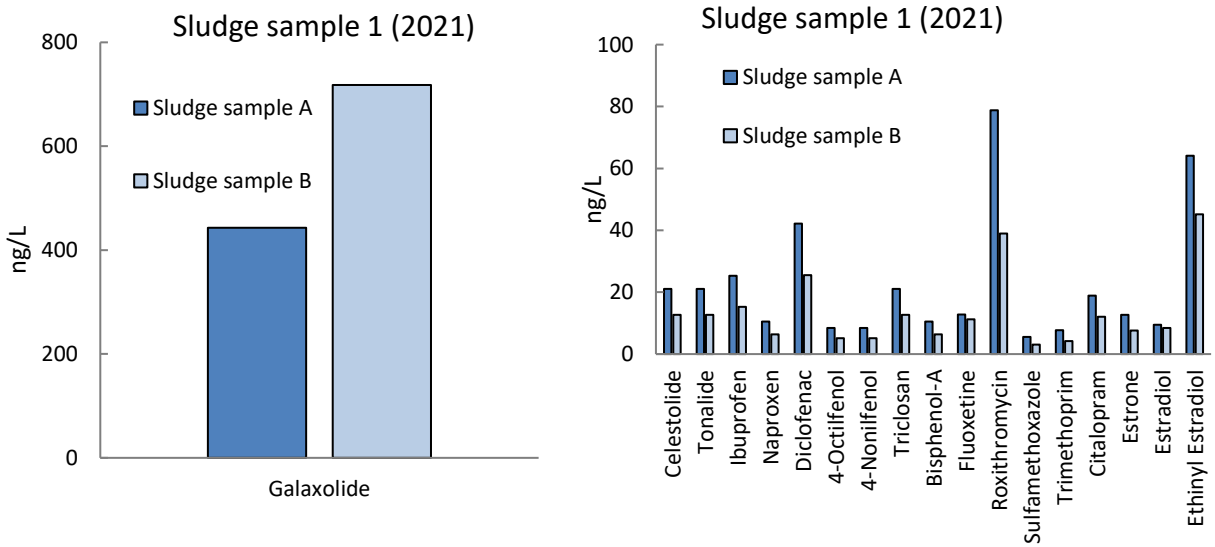
Figure 12: Black water samples in 2021.

In this case, in addition to the MPs present in the previous samples (Ibuprofen, Naxoprene and Galaxolide), Fluxetine and Citalopram (belonging to antidepressant drugs) as well as Estrone (natural oestrogens) were measured.

The presence of micropollutants in black water, which are more commonly observed in grey water, is due to persistent MPs in grey water end up in the black water as a consequence of the refilling of the cisterns.

Sludge:

In 2021, several anaerobic sludge samples were carried out: A and B samples were carried out in March and C and D samples in May.



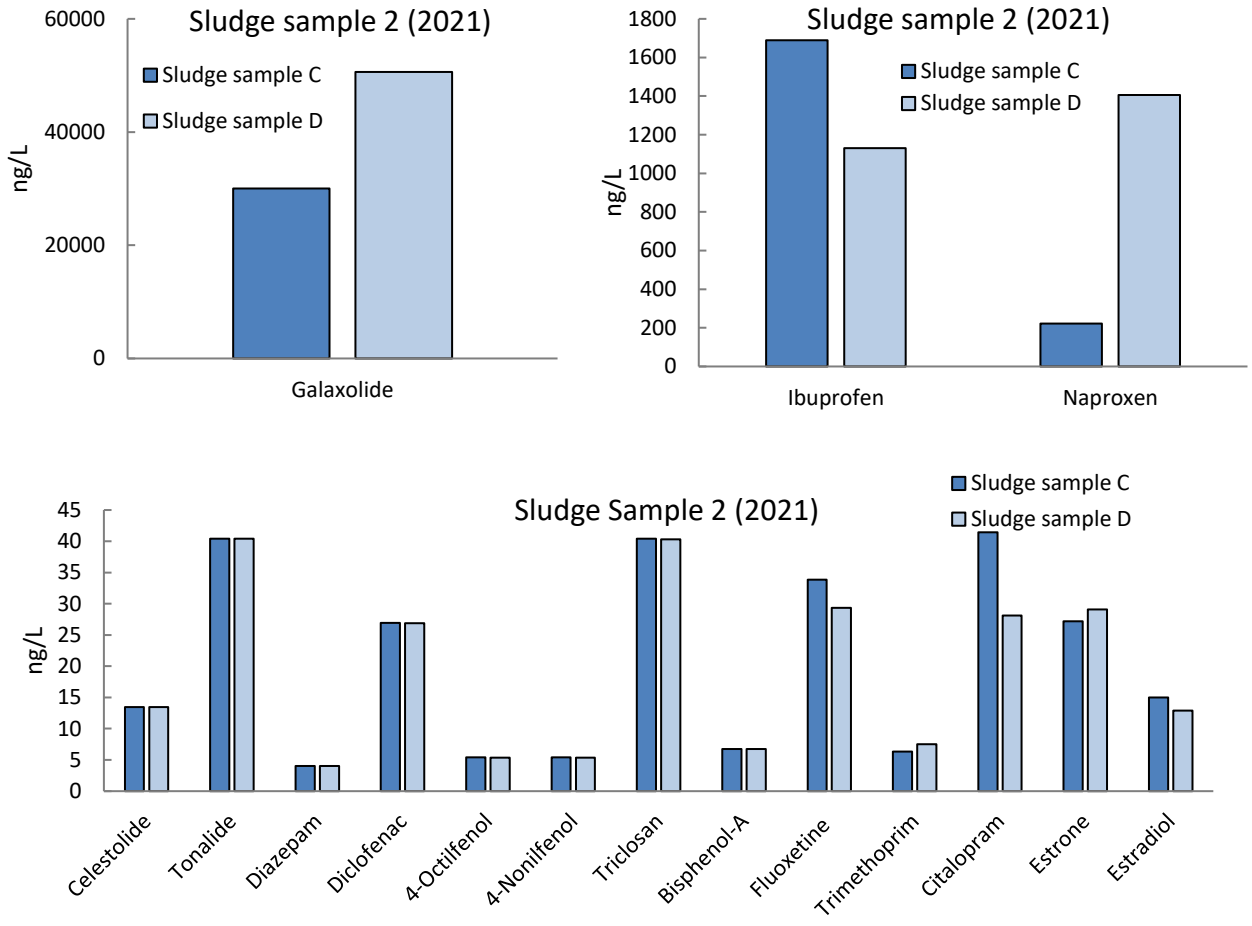


Figure 13: Grey water samples in 2021

Polycyclic fragrances and non-steroidal anti-inflammatories are the most abundant compounds detected in Porto do Molle sludge. This stream is the endpoint of most hydrophobic pollutants through sorption (Carballa et al., 2008), but also of an important fraction of hydrophilic MPs not bio transformed during the wastewater treatment. The concentrations of MPs in sewage sludge are much dependent on their physicochemical characteristics and usage rates.

These results are very similar to the ones obtained in other studies (Stasinakis, 2012) where in general, hydrophobic substances, such as triclosan and musk fragrances, are detected at important concentrations (up to 10 000 mg/kg), while much lower levels (10-100 mg/kg) are measured for hydrophilic pharmaceuticals, as diclofenac, trimethoprim, ibuprofen, naproxen, carbamazepine or sulfamethoxazole.

3.2.2 Virus

The application of MBR technology in municipal and industrial wastewater treatment has rapidly expanded with an average global market growth of over 10% since the turn of the millennium (Santos et al., 2011), as the result of more stringent environmental regulations, as well as the various advantages AnMBR provides compared to conventional treatment processes, including high effluent quality, reduced environmental footprint, and nutrient recovery.

Effective management of WWTP requires recognition that virus concentration in influent will vary – particularly in small and medium plants. Irrespective of treatment type, culturable viruses are likely to be present in non-disinfected effluent, with associated human health risks dependent on concentration and receiving water usage.

The way in which the types of pathogens are classified depends on the taxonomic category to which they belong. According to their structural level, pathogens are classified as shown in Table 3:

Table 3: taxonomic category pathogens

TAXONOMIC CATEGORY	SIZE	PROPAGATION AREA
PRIONS	<20 nm	Intracellular
VIRUSES	20-400nm	Intracellular bound
BACTERIA	0.2-15 µm	Intracellular bound Extracellular Intracellular facultative
FUNGUS	2-200 µm	Extracellular Intracellular Facultative
PROTOZOA	1-50 µm	Extracellular Intracellular facultative Intracellular bound
HELMINTHS	3 mm-10 m	Extracellular Intracellular

Viruses are microscopic entities that have a core of genetic material, either DNA or RNA. The core is covered with a capsid, a protective coat made of protein. DNA viruses are mostly double stranded, while RNA viruses are single-stranded. An RNA virus is a virus which has ribonucleic acid (RNA) as its genetic material. The nucleic acid is usually single-stranded RNA (ssRNA), but it may be double-stranded RNA (dsRNA)

Caliciviruses are small (27-35 nm in diameter), non-enveloped, icosahedral viruses with positive-sense, single-stranded RNA genomes (7.4 to 8.3 kb in size). This family is divided into different genera, two of which infect humans: norovirus (NoV) and sapovirus (SaV). Both NoV and SaV are genetically diverse and divided into five genogroups (G). For NoV, three genogroups (GI, II and IV) infect humans) while for SaV, all genogroups infect humans except GIII. These viruses are shed at high concentration (up to 1011 particles/g) in faeces during the acute phase of the disease and up to three weeks after symptoms have subsided. Shedding in asymptomatic, infected individuals has also been observed. As a consequence, these viruses are detected in high concentration in human sewage.

Both NoV and SaV are transmitted via the faecal-oral route and belong to the most infectious group of causative agents of epidemic gastroenteritis. If wastewater treatment is not efficient, these viruses can persist for a long time in the environment and may contaminate coastal or surface waters as they are very resistant.

The methodology used for viruses' analysis was:

- Virological analysis was performed using specific RT-qPCRs.

- Viral RNA was extracted from two independent subsamples of each sample and subjected to two independent amplifications. In all cases the positive controls, both extraction and amplification, showed specific amplification, thus ruling out the possible existence of inhibitors that could alter the result. No amplification was obtained in any negative controls, ruling out any possible cross contamination.

It is important to highlight that of the input samples analysed in which is not detected a presence of virus, it is not analysed at the output, since its presence would not make sense in that case

Grey water:

Table 4 shows a summarized table with viruses detected in grey water samples.

Table 4: Virus in grey water samples.

		NoV GI (ARN/L Copies)			NoV GII (ARN/L Copies)			SaV (ARN/L Copies)		
GREY WATER		GW inlet	GW outlet	BW outlet+Chlor.	GW inlet	GW outlet	BW outlet+Chlor.	GW inlet	GW outlet	BW outlet+Chlor.
Sample 1	03/03/2020	ND	ND	ND	ND	ND	ND	ND	ND	ND
	10/03/2020	ND	ND	ND	ND	ND	ND	ND	ND	ND
Sample 2	30/11/2020	ND	NA	NA	ND	NA	NA	ND	NA	NA
	14/12/2020	1.99E+03	ND	ND	3.00E+02	3.97E+04	ND	ND	NA	NA
Sample 3	24/05/2021	ND	NA	NA	ND	NA	NA	ND	NA	NA
	07/06/2021	5.00E+03	ND	ND	3.38E+04	ND	ND	ND	NA	NA

(NA: Not analysed, ND: No detected).

Norovirus G1 and G2 (Nov GI and Nov GII) was detected in Grey Water inlet stream in two of three samples.

These viruses were no detected after passing though the membrane or after disinfection with chlorine, so we can affirm the effectiveness of the membrane treatment for virus elimination. No presence of Sapovirus (SaV) was observed in any sample in greywater.

Black water:

Table 5 shows a summarized table with viruses detected in black water samples.

Table 5: Virus in blackwater samples

		NoV GI (ARN/L Copies)			NoV GII (ARN/L Copies)			SaV (ARN/L Copies)		
BLACK WATER		BW inlet	BW outlet	BW outlet+U.V	BW inlet	BW outlet	BW outlet+U.V	BW inlet	BW outlet	BW outlet+U.V
Sample 1	03/03/2020	ND	ND	ND	ND	ND	ND	1.69E+06	4.01E+03	5.47E+02
	10/03/2020	ND	ND	ND	ND	ND	ND	7.83E+05	7.77E+04	2.40E+04
Sample 2	30/11/2020	3.48E+02	ND	7.22E+03	ND	ND	ND	ND	4.04E+04	8.73E+03
	14/12/2020	ND	4,57E+03	ND	ND	3.93E+04	5.55E+04	ND	ND	ND
Sample 3	24/05/2021	4.70E+04	ND	ND	ND	ND	ND	ND	ND	ND
	07/06/2021	7.21E+04	1.44E+05	1.13E+03	1.96E+03	7.16E+03	4.82E+02	ND	ND	ND

Sample 4	02/07/2021	3.39E+01	ND	NA	3.56E+01	ND	NA	NA	NA	NA
Sample 5	31/08/2021	3.94E+01	ND	NA	3.15E+01	4.03E+01	NA	NA	NA	NA
Sample 6	28/09/2021	4.05E+01	ND	NA	2.77E+01	3.50E+01	NA	NA	NA	NA

. (NA: Not analysed, ND: No detected)

The results of virus presence in black water inlet stream reveals existence of Nov G1, Nov G2 and SaV. Sample 2 shows an incoherent result that can be explained due to a sampling punctual contamination. In sample 3 can be deduced a correct but an insufficient effectiveness membrane performance. The effectiveness of the U.V disinfection system used is correct.

Sars-Cov2:

In addition to these viruses, and taking account the current pandemic situation, it was decided to also analyse the SARS-CoV-2 virus. Coronaviruses are a family of a linear single-stranded positive RNA genome, named for the crown-like spikes on their surface that can widely spread in humans, other mammals and birds, and cause diseases related to respiratory tract infections, intestinal, liver and nervous system.

The methodology used for viruses' analysis was:

- Virological analysis was performed using specific RT-qPCRs and in the case of SARS-CoV-2, the polymerase (IP4) and nucleocapsid (N1) genes.
- Viral RNA was extracted from two independent subsamples of each sample and subjected to two independent amplifications. In all cases the positive controls, both extraction and amplification, showed specific amplification, thus ruling out the possible existence of inhibitors that could alter the result. No amplification was obtained in any negative controls, ruling out any possible cross contamination.

SARS-CoV-2 was not analysed in sample nº 1, due to the fact that at the beginning of March 2021, pandemic situation was going to happen and were still no aware the importance of detecting SARS-CoV-2 ARN copies in wastewater (Table 6).

Table 6: SARS-CoV-2 Virus in grey water and black water

		SARS-CoV-2 (ARN/L Copies)		
GREY WATER		GW inlet	GW outlet	BW outlet+Chlor.
Sample 1	03/03/2020	Before COVID Lockdown		
	10/03/2020			
Sample 2	30/11/2020	ND	ND	ND
	14/12/2020	ND	ND	ND
Sample 3	24/05/2021	NA	NA	
	07/06/2021	NA	NA	

		SARS-CoV-2 (ARN/L Copies)		
BLACK WATER		BW inlet	BW outlet	BW outlet+U.V
Sample 1	03/03/2020 10/03/2020	Before COVID Lockdown		
Sample 2	30/11/2020	ND	ND	ND
	14/12/2020	ND	ND	ND
Sample 3	24/05/2021	ND	ND	
	07/06/2021	ND	ND	
Sample 4	02/07/2021	ND	ND	
Sample 5	31/08/2021	ND	ND	
Sample 6	28/09/2021	ND	ND	

3.2.3 Other pathogens

This report evaluates the applicability of the reclaimed water in agriculture as irrigation water with nutrients, and possible uses in industry or in the buildings, according to reclaimed water quality regulation (EU)2020/741 of 25 May 2020 on minimum requirements for water reuse

In order to know the pathogens content in samples and the applicability of the re-claimed water in agriculture as irrigation water, an accredited laboratory was subcontracted. The contract included analytics and reference method of measurement shows in Table 7

Table 7: Physico-chemical, microbiological and hydrobiological parameters

PHYSICO-CHEMICAL PARAMETERS	
BDO $_5$	Electrometry
SUSPENDED SOLIDS	Gravimetry
TURBIDITY	Nephelometry
MICROBIOLOGY	
LEGIONELLA.	Detection and counting
TOTAL COLIFORMS (COUNT)	Membrane filtration
FECAL COLIFORMS (COUNT)	Membrane filtration
ESCHERICHIA COLI (COUNT)	Membrane filtration
HYDROBIOLOGY	
NEMATODE EGGS	Microscopy (*)

(*)Method based on "Analysis for Use in Agriculture-A laboratory manual of parasitological and bacteriological techniques" Ayres & Mara O.M.S (1996).

Reclaimed water quality classes and the permitted uses and irrigation methods for each class are set out in Table 8 and *Table 9*.

Table 8: Classes of reclaimed water and permitted agricultural use and irrigation methodsreclaimed water quality regulation (EU)2020/741 of 25 may 2020)

Minimum reclaimed water quality class	Crop category (*)	Irrigation methods
A	All food consumed raw where the edible part is in direct contact with reclaimed water and roots crops consumed water	All irrigation methods
B	Food crops consumed raw where the edible part is produced above ground and is no in direct contact with reclaimed water processed food crops and non-food crops including crops used to feed milk or meat producing animals.	All irrigation methods
C	Food crops consumed raw where the edible part is produced above ground and is no in direct contact with reclaimed water, processed food crops and non-food crops including crops used to feed milk or meat producing animals	Drip irrigation (**) or other irrigation method that avoids direct contact with the edible part of the crop
D	Industrial, energy and seeded crops	All irrigation methods (***)

(*)If the same type of irrigated crop falls under multiple categories of Table 1, the requirements of the most stringent category shall apply.

(**) Drip irrigation (also called trickle irrigation) is a micro-irrigation system capable of delivering water drops or tiny streams to the plants and involves dripping water onto the soil or directly under its surface at very low rates (2–20 litres/hour) from a system of small-diameter plastic pipes fitted with outlets called emitters or drippers.

(***) In the case of irrigation methods which imitate rain, special attention should be paid to the protection of the health of workers or bystanders. For this purpose, appropriate preventive measures shall be applied.

Table 9: Quality requirements for reclaimed water class reclaimed water quality regulation (EU)2020/741 of 25 May 2020)

Reclaimed water quality class	Indicative technology target	Quality requirements				
		E.coli **(number/100 ml)**	**BOD_5 (mg/L)**	**TSS (mg/L)**	**Turbidity (NTU)**	**Other**
A	Secondary treatment, filtration and disinfection	≤ 10	≤ 10	≤ 10	≤ 5	*Legionalle spp*: <1 cfu/L where there is a risk of areosolisation intestinal neamtodes (helminth eggs); ≤ 1 egg/L for irrigation of pastures or forage
B	Secondary treatment and disinfection	≤ 100	In accordance with Directive 91/271/ECC, Annex I, Table 1	In accordance with Directive 91/271/ECC, Annex I, Table 1	-	
C	Secondary treatment and disinfection	≤ 1000			-	
D	Secondary treatment and disinfection	≤ 10000			-	

In accordance to Directive 91/271/CE of quality requirements for BOD_5 and TSS concentration requirements in B, C and D reclaimed water quality class shows Table 10.

Table 10: Directive 91/271/CE Quality requirements.

Quality requirements	**Directive 91/271/CE (Annex I-Table 1)**		
Parameters	**Concentration**	**Minimum percentage of reduction (%) (*)**	**Reference method of measurement**
BOD_5 (mg/L) (1)	**25** mg/L O_2	**70-90** (**40** under Article 4)	Homogenized, unfiltered undecanted sample. Determination of dissolved oxygen before and after five-day incubation at 20ºC ± 1ºC, in complete darkness. Addition of a nitrification inhibitor
Chemical Oxygen demand (COD)	**125** mg/L O_2	**75**	Homogenized, unfiltered, undecanted sample. Potassium dichromate
TSS (mg/L)	**35** mg/L (under Article 4-more than 10.000 pe) or **60** mg/L (for 2000-10 000 pe)	**90** (for more than 10 000 pe) and **70** (for 2 000 to 10 000 pe)	-Filtering of a representative sample through a 0,45 µm filter membrane. Drying at 105ºC and weighing. -Centrifuging of a representative sample (for at least five mins with mean acceleration of 2800 to 3200 g) drying al 105 and weighing

(*) Reduction in relation to the load of the influent.

(1) The parameter can be replaced by another parameter: total organic carbon (TOC) or total oxygen demand (TOD) if a relationship can be established between BOD_5 and the substitute.

Since 2021, several samplings were taken from the following process streams (Table 11 and Table 12):

- Greywater inlet (GW inlet): which corresponds to the water generated by the handwashing
- Greywater outlet (GW outlet): is treated water after filtered process

- Blackwater outlet (BW outlet): is treated water after AnMBR process
- Blackwater after disinfection (BW outlet +U. V): is water stream after disinfected treatment

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 730285.

Grey water:

Table 11: Greywater pathogens content

Date	Quality Class	Quality Class	*E. coli* UFC/Volume (ml)		*Legionella* u.f.c./L		Helminth eggs (ud/10 L)		NTU		DBO_5 (mg/L)		TSS (mg/L)	
	Before MBR	After MBR	Before MBR	After MBR	Before MBR	After MBR	Before MBR	After MBR	Before MBR	After MBR	Before MBR	After MBR	Before MBR	After MBR
22-7	-	A	7000	<1 / 10 ml	ND	<1	<1	<1	3.21	<0.2	43.7	<5	7.8	<2
24-8	-	A	<1/0.00001 ml	<1/10	ND	<1	<1	<1	10.9	<0.2	67.8	<5	35.8	<2
7-9	-	A	8000	1-3 /100 ml	ND	<1	<1	<1	12.5	<0.2	27.4	<5	25.4	<2
23-9	-	A	100-300 /100 ml	<1	ND	<1	<1	<1	3.73	<0.2	14.1	<5	7.09	2,95
7-10	-	A	<1/0.1 ml	<1/100	ND	<1	<1	<1	6.33	<0.26	18.4	<5	9.96	<2
21-10	-	B	4.00E+03	4.40E+01	<1	<1	<1	<1	2.94	0.26	<5	<5	29.2	<2

Although it is showing the results obtained for the water inlet and outlet streams, only grey water outlet stream has been classified in accordance with current regulations. It is important to note that the water did not pass through the existing chlorine disinfection system.

The results for pathogens show that reuse water meets the requirements for type A quality (maximum quality) in all the samples taken, except for one sample that meets the requirements for type B quality. So, this effluent it allows to use in agricultural for all foods consumed raw where the edible part is in direct contact with reclaimed water and roots crops consumed water in all irrigation methods.

Therefore, we can say that reuse grey water for would comply correctly with the required limits without the need to carry out the disinfection treatment mentioned above.

This project has received funding from the European Union's Horizon 2020 research and innovation programme under grant agreement No 730285.

Black water:

Table 12: Pathogens content in blackwater

Date	Quality Class	Quality Class	*E. coli* UFC/Volume (mL)		Legionella (u.f.c./L)		Helminth eggs (ud/10 L)		NTU		DBO5 (mg/L)		TSS (mg/L)	
	Before UV	After UV	Before UV	After UV	Before UV	After UV	Before UV	After UV	Before UV	After UV	Before UV	After UV	Before UV	After UV
26-1	A	-	0		ND	ND	<1		<0.25		6.7		<10	
17-3	B	B	8	14	ND	ND	<1		0.89	0.59	11.6	25	<10	<10
30-4	A	B	<1 / 0.01 ml	<1 / 0.01 ml	<1	<1	<1	<1	0.91	1.4	9.88	7.85	8.06	15
12-5	B	B	<1 / 0.1 ml	<1 / 0.1 ml	ND	ND	<1	<1	5.12	6.57	22.4	NA	6.06	<5.63
16-6	A	B	<1 / 0.1 ml	<1 / 0.1 ml	<1	<1	<1	<1	1.89	14	9.03	14.3	< 2	< 2
1-7	C	D	100-300 ufc/100 ml	1000-3000 ufc/100 ml	<1	<1	<1	<1	0.47	0.89	14.5	9.87	< 2	< 2
4-8	D	D	1000-3000 ufc/1000 ml	1000-3000 ufc/1000 ml	<1	<1	<1	<1	0.51	7.22	11.8	14.6	< 2	< 2

Date	Quality Class	Quality Class	*E. coli* UFC/Volume (mL)		Legionella (u.f.c./L)		Helminth eggs (ud/10 L)		NTU		DBO5 (mg/L)		TSS (mg/L)	
	Before UV	After UV	Before UV	After UV	Before UV	After UV	Before UV	After UV	Before UV	After UV	Before UV	After UV	Before UV	After UV
18-8	B	B	<1/1 ml	<1/1 ml	<1	<1	<1	<1	61.5	48.9	8.32	11.2	<2	<2
31-8	B	B	<1/0.1 ml	<1/0.1 ml	<1	<1	<1	<1	<2	<2	10.6	13	<2	<2
14-9	B	B	<1/0.1 ml	<1/1 ml	<1	<1	<1	<1	<0.20	0.33	14	14.4	<2	<2
30-9	B	D	<1/0.1 ml	1000-3000 ufc/100 ml	ND	ND	<1	<1	0.86	1.41	23.7	10.4	7.78	<5

The results for pathogens show that permeate meet Type B quality requirements in 70% of samples taken which it allows all irrigation methods in agricultural use for food crops consumed raw where the edible part is produced above ground and is no in direct contact with reclaimed water processed food crops and non-food crops including crops used to feed milk or meat producing animals.

Pathogen removal capacity was been less than expected despite AnMBR operation, hypochlorite cleanings were carried out in the pipe's effluent following and in the permeate pump. Subsequently, the pump was replaced, and a new LED lamp was placed. The results after these modifications improved the quality of the permeate keeping it within the parameters allowed for type B quality. (parameter data available only after publishing this report).

3.2.1 Fertigation pot tests

Experimental layout

The effluent from the AnMBR unit treating source separated blackwater in Vigo demoplant was used as a fertigation water in pot experiments cultivating ray grass and basil. The experiments were performed in Sweden and in Spain using the same batch of seeds and mineral fertilisers for controls, whereas it used different soils available at the sites for cultivation as well as different batches of AnMBR effluent (Table 13). The crops were at each location cultivated in two types of soils and the fertigation water was applied according to the irrigation need of the plant, either as a sole source of nutrients or in combination with mineral fertiliser.

Mineral fertilisers applied were either as NPK (Nitrogen, Phosphorus, Potassium) but also as a blend with a larger range of macro- and micronutrients to ensure no nutrient deficiencies in the controls. Similar controls irrigated with deionised water was studied for comparison. At harvest the wet and dry biomass was measured, but only dry mass is presented in this report. Only a first cut of ray grass was harvested. The conditions for the pot experiment was rather different with experiment 1 performed outdoors also subjected to rain, whereas experiments 2 and 3 was performed in green houses however with differences in temperature and light intensity.

Table 13: Outline of the three pot experiments

	Location	Plants	Controls N-P-K-Ca-S-Mg (kg/ha)	Irrigation (mm)	ferti. (kg N/ha)	AnMBR NH-N (g/L)	Soils
1	Sweden, Uppsala	Grass	120-27-124-207-52-0	-	-	0.11	Sand; Clay
2	Sweden, Uppsala	Grass	158-22-90-90-104-42*	192	212	0.052	Sand; Peat based with clay
		Basil	274-80-275-180-173-80*	94	49		
3	Spain, Salceda	Grass	120-22-125-90-120-42	473	688	0.12	Sand; Clay
		Basil	100-38-131-86-95-38	555	587		

a,) Micronutrients was given as a composite micronutrient solution based on iron (Fe) application rate 3.75 kg /ha resulting in 0.25 Cu, Zn and Mn, 0.60 B and 0.060 Mo in kg/ ha).

In experiment 2 an error was done when mixing the control blend and thus the nitrogen application rate became higher than intended and for the grass the K and S became lower than intended. Thus the ratio between

macronutrients varied between experiments (Table 13). The fertigation water had an ammonia nitrogen (NH-N) concentration of 0.052-0.012 g N/L. When analysing both total nitrogen and ammonia nitrogen (Exp 1 and 2) approximately 10% of the total nitrogen was bound in organic nitrogen and may act as a slow release nitrogen source. Applying the fertigation water according to the irrigation need resulted in very different volumes applied to the different crops and the nitrogen application by fertigation did for grass in all experiments exceed the dose given by mineral fertilisers as controls (Figure 14). Adding a wider range of macro nutrients than NPK as well as micronutrients by mineral fertilisers did only in one case result in higher yield (Grass on sand in Exp 1) and then only in combination with fertigation, thus the results presents the NPK controls.

Crop physical appearance

The ray grass plants with fertigation showed no signs of nutrient deficiencies whereas the plants cultivated with no fertiliser and irrigated with water were paler and less dark green indicating nutrient deficiency (Figure 14). For plants receiving only NPK fertiliser the grass plants were in experiment 3 (but not in experiment 2) slightly paler than the grass receiving only fertigation water or NPK plus fertigation water. This could maybe be related to that the dose of NPK was lower in experiment 3 than in 2 but also to that in experiment 3 the crop period was longer (56 days compared to 41 days) so grass plants grown for a longer period would also require more nutrients. For the Basil the plant appearance indicated nutrient deficiencies when grown with no fertilisation but also when receiving NPK mineral fertiliser, most notable in the third experiment where the mineral fertiliser were applied at a lower dose as well as having a longer cropping period before harvest than in the second experiment (Figure 15).

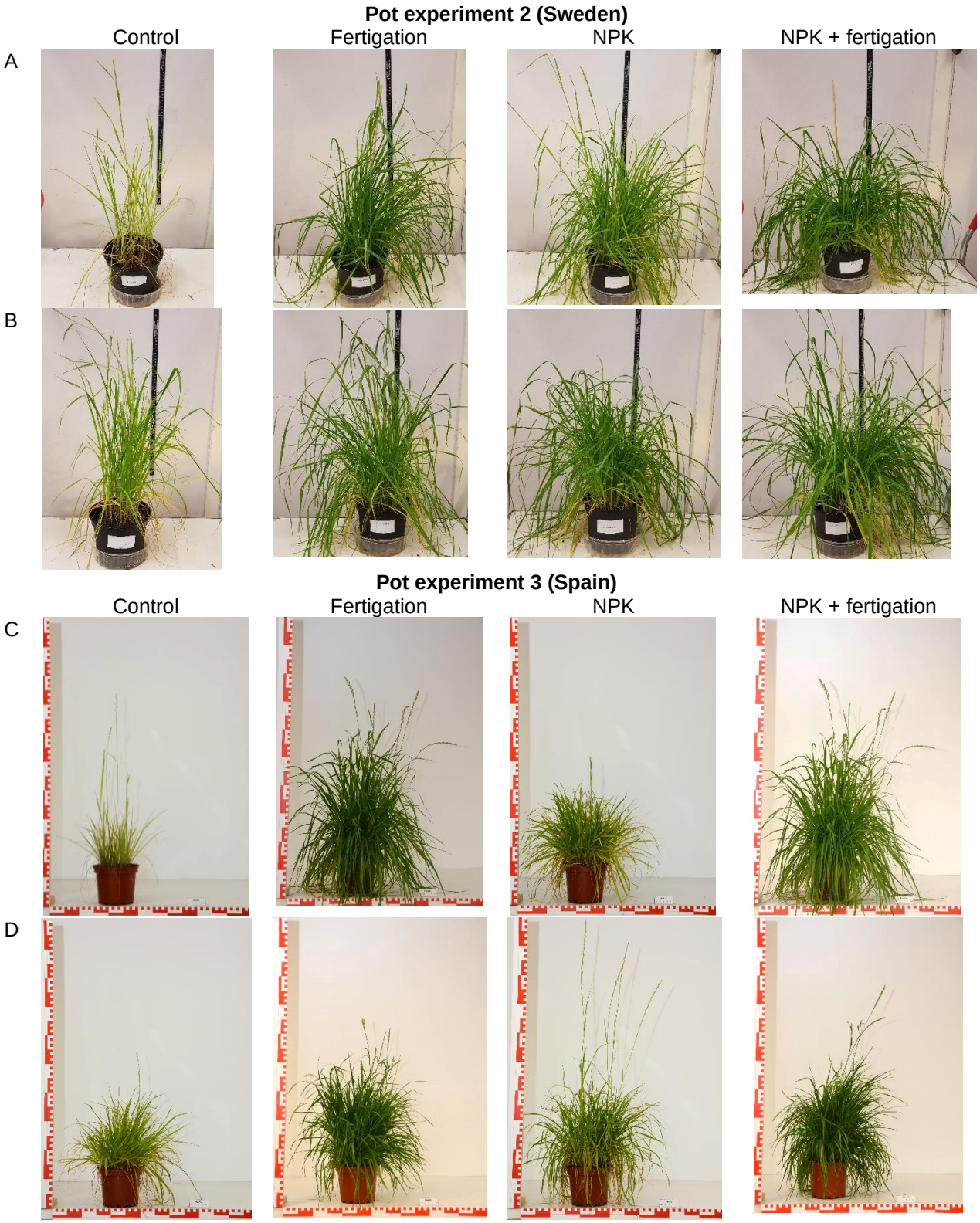


Figure 14: Ray grass plants from pot experiments 2 and 3 at the state of harvest (day 41 and 56 respectively) cultivated on sand (row A and C) and on clay (row B and D). A selection of a representative photo from the replicates were used.

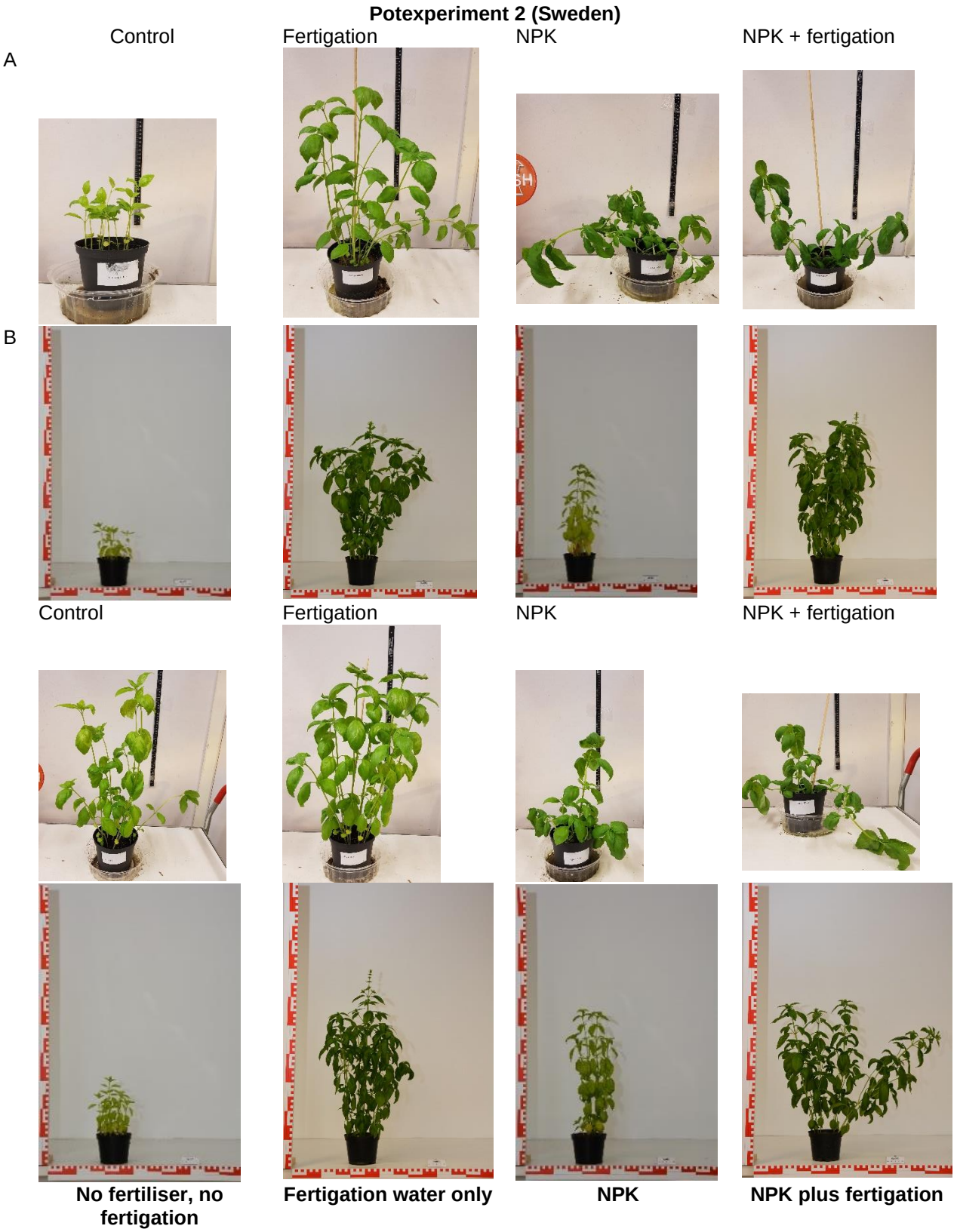


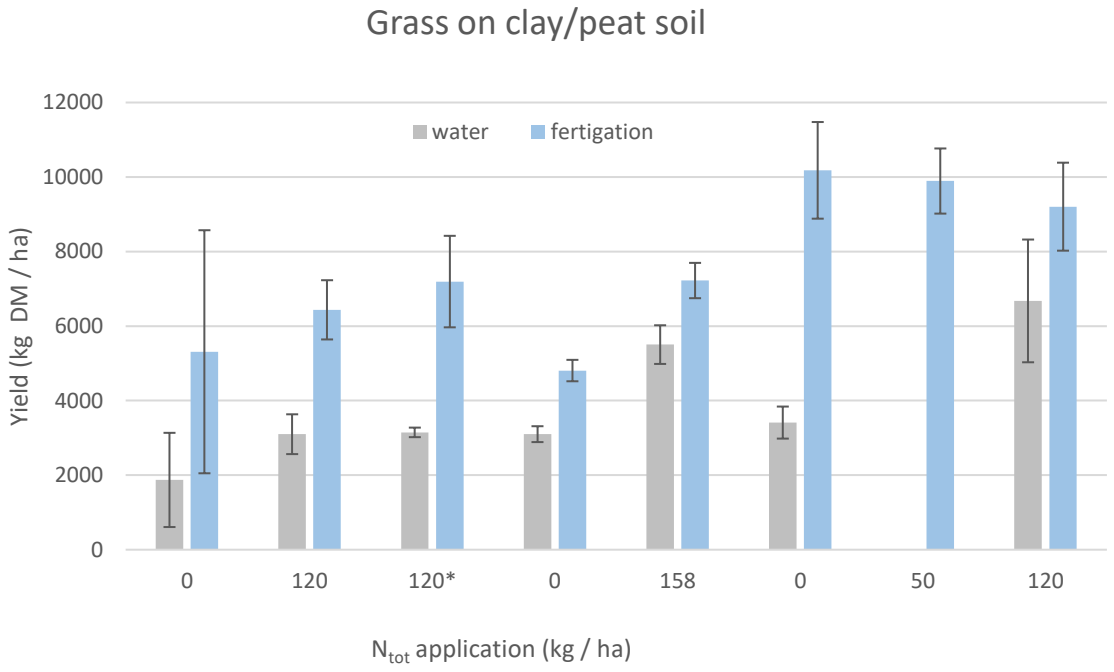
Figure 15: Sweet basil plants from pot experiment 2 and 3 at the state of harvest (day 41 and 56 respectively) cultivated on sand (row A and B) and on clay (row C and D). A selection of a representative photo from the replicates were used.

Dry matter yields

Due to those experiments were undertaken with different soils and under different climatic conditions the yield produced with the same mineral fertiliser application varied largely and with mineral fertilisation application 120 kg N per ha the resulting dry matter yield were around in 3100 kg DM in experiment 1 and 6680 kg in experiment 3 (Table 14). For the ray grass that was cultivated under different conditions in three experiments the overall trend was (even though not always significantly different) lower average yield on the sand soil compared to clay/peat soil and that fertigation gave an increased average yield compared to similar setup irrigated with water (Figure 16). The yield of the grass could partly be related to the application rate of nitrogen. The increase in yield when fertigating was larger on sand than on clay/peat, up to 8.5 times higher compared to unfertilised soil irrigated with deionised water (Table 14).

Table 14: Ray grass yield (Kg dry matter/ha) cultivated on clay/peat or sand soil and irrigated with deionised water or fertigation water. Yield increase in brackets was not statistically significant (alpha 0.05)

		Experiment 1			Experiment 2		Experiment 3		
		N_0	N_{120}	N_{120}+	N_0	N_{158}	N_0	N_{60}	N_{120}
Clay/peat soil	water	1872	3099	3148	3100	5504	3411		6676
	fertigation	5312	6437	7195	4807	7223	10180	9893	9205
	Yield increase fertigation/water	*(2.8)*	*2.1*	*2.3*	*1.6*	*1.3*	*3.0*		*(1.4)*
Sand soil	water	208	863	811	1034	4912	841		8037
	fertigation	1740	3280	3662	3588	5578	7160	5431	10054
	Yield increase fertigation/water	*8.4*	*3.8*	*4.5*	*3.5*	*(1.1)*	*8.5*		*(1.3)*



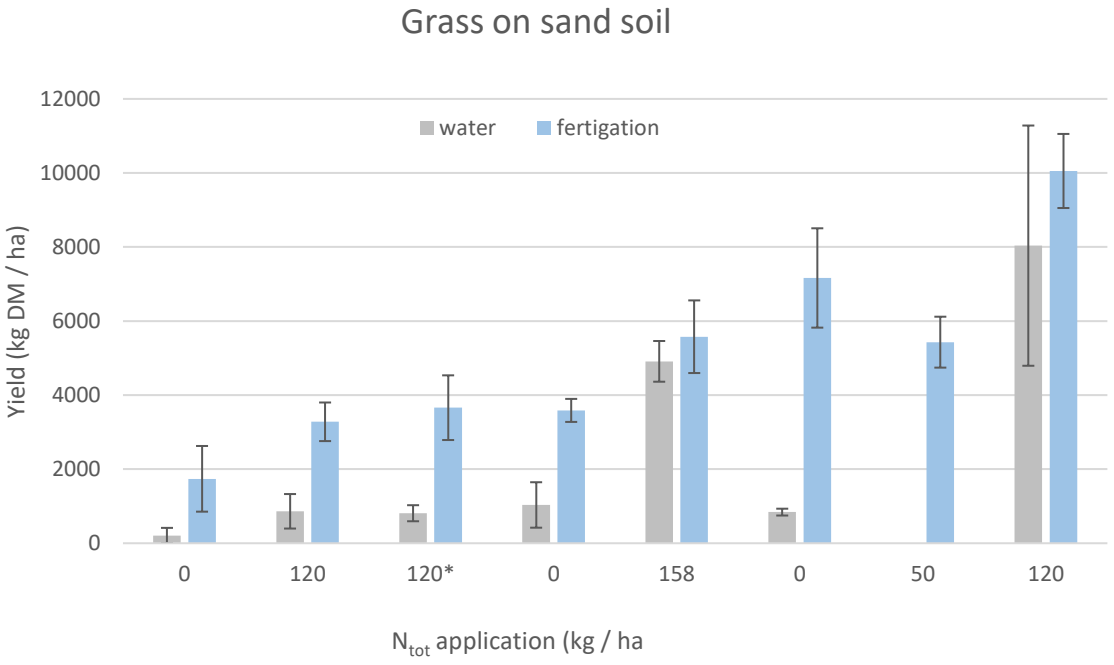


*Figure 16: Crop yield (kg DM/ ha) for Ray grass on clay (upper pane) and on sand (lower pane) with grey bars showing yield when receiving water for fertilisation and blue bars showing yield when receiving fertigation with the initial base fertilization being the same (0-160 kg N/ha).Error bars show the standard deviation of the mean. Asterisk * indicate that in addition to NPK also more macronutrient and micro nutrients were applied.*

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Table 15: Sweet basil yield (kg dry matter/ha) cultivated on clay/peat or sand soil and irrigated with deionised water or fertigation water. Yield increas in brackets were not statistically significant (alpha 0.05)

		Experiment 2		Experiment 3		
		N0	**N275**	**N0**	**N50**	**N100**
Clay/peat soil	water	3020	2251	2620	-	7560
	fertigation	3448	1137	9673	11515	12417
	Yield increase fertigation/water	*(1.1)*	*(0.5)*	*3.7*	-	*1.6*
Sand soil	water	330	1817	694	-	9068
	fertigation	1629	727	12579	11564	9641
	Yield increase fertigation/water	*(4.9)*	*(0.4)*	*18.1*	-	*1.4)*

Also for the sweet basil dry matter yield the similar observations could be done as for the ray grass. Fertigation did for almost all cases result in higher yield compared to the same treatment receiving water for irrigation, however for some cases not statistically significant differences. In the case when basil plants had received an initial NPK dose at start in experiment 2 the use of fertigation water resulted in less than half the yield. Due to the high NPK fertiliser application rate (275 kg N/ha) the fertigation did probably give deleterious concentrations of some nutrient. This is supported by the fact that when sweet basil was cultivated on the rather nutrient rich clay/peat soil the controls gave higher yield compared to the fertilised plants. Interestingly, when fertigation was used in the experiment 3 the water need of the plants resulted in very high application of nitrogen, around 600 kg NH-N/ ha, resulting in among the highest yields and dry matter yield were not affected by the initial dose of mineral fertiliser (50 or 100 kg N/ha) (Figure 17).

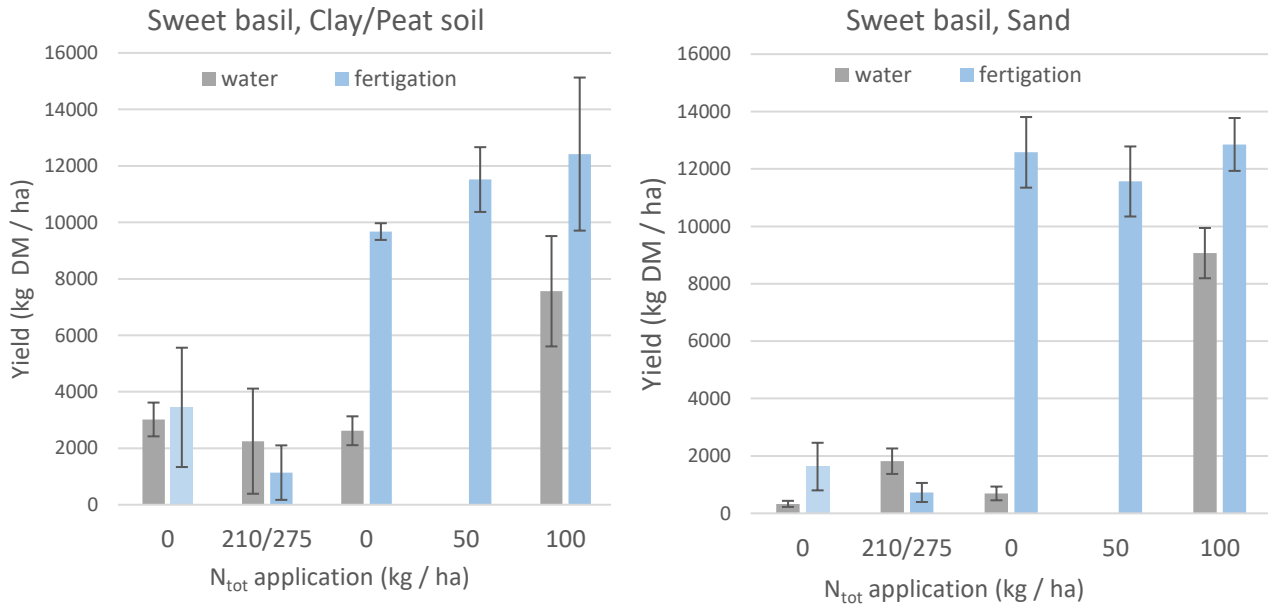

Figure 17: Crop yield (kg DM/ ha) for Sweet Basil on clay (left pane) and on sand (right pane) with blue bars showing yield when receiving water and green bars showing yield when receiving fertigation with the initial base fertilization being the same (0-160 kg N/ha). .Error bars show the standard deviation of the mean

The crop response to total nitrogen application show that even a larger total application was done with the fertigation water (yellow round symbols Figure 18) compare to mineral fertiliser (blue round symbols Figure 18) the yield was not necessarily larger indicating that the timeliness of the application of nutrients was not optimal and that plant growth may have been limited by initial low rates as application of fertigation was based on irrigation need. Thus, it seems to be beneficial to combine fertigation with initial fertilization. However, in the 3rd experiment, where large volumes of fertigation water were applied, the combined fertigation and mineral fertilization yielded less or similar than fertigation alone (square symbols) indicating that with large irrigation volumes the optimal nutrient application may be exceeded, and the excess nutrients hamper the growth (Figure 18).

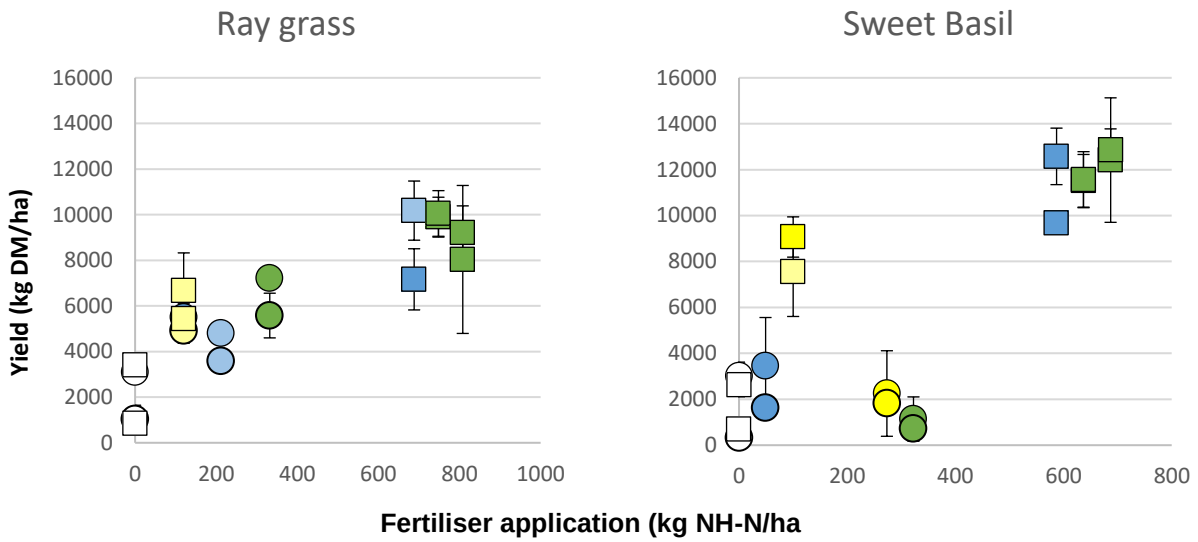

Figure 18: Yield plotted against application of total nitrogen for experiments 2 and 3 with white symbols receiving no fertiliser/fertigation, yellow receiving mineral fertiliser only, blue receiving fertigation water only and green receiving a combination of mineral fertiliser and fertigation water. Round symbols depict results from Exp. 2 and square symbols results from Exp.3. .Error bars show the standard deviation of the mean The lower yield is always the average from cultivation on sand.

In conclusion over the three experiments conducted with different soils and climates, for ray grass the fertigation always contributed to the crop yield and could probably under circumstances of high irrigation need be the sole source of nitrogen. On nutrient poor soil plants would probably benefit from an intial fertilisation so that nutrients available match the early crop need. Fertigation is suggested to not be combined with full fertiliser application rates since its very probable that the optimal nutrient application will be exceeded. Initial fertilization needs to consider, in addition to crop nutrient needs, the projected volumes of fertigation that will be used. Another approach could also be that fertigation is done according to fertiliser need and is combined with regular irrigation when at risk of exceeding optimal nutrient dose for plant growth.

3.2.2 Toxicity tests

Seed germination/Root elongation toxicity test (OPPTS 850.4200 [1]) and the Earthworm, Acute Toxicity Tests (OECD 207 [2]; filter paper contact test) were performed for samples received from Vigo and Ghent demo plants at different sampling campaigns (2019, 2020, and 2021).

The test vial used for both tests was a plastic Petri dish of 9 cm diameter and 2 cm height, containing filter paper at the bottom for support. Six concentrations were prepared by dissolving each sample in deionized water in a geometric series (3.12, 6.25, 12.5, 25, 50 and 100%), and 5 mL was pipetted into each Petri dish to wet the filter paper. Blank tests were performed with 5 mL of deionized water only. The pH and electrical conductivity of all sample's concentrations were determinate before starting the test.

Seed germination/Root elongation toxicity test

The phytotoxicity was evaluated by the seed germination technique, with seeds of lettuce (*Lactuca sativa*, variety Romana). Two replicates of 15 seeds were placed in each Petri dish, after the application of 5 ml of sample or distilled water (control), the dishes were incubated for 5 days at 22 ± 1ºC and light cycled in 16:8 h light:dark periods. The relative seed germination, relative root elongation and Germination Index (GI) were calculated using the following equations (Tiquia and Tam, 1998 [3]):

Relative root elongation (%) = Mean root length with sample / Mean root length with control × 100 (1)

Relative seed germination (%) = Seeds germinated with sample / Seeds germinated with control × 100 (2)

Germination Index (GI) = (% seed germination * % root elongation) /100 (3)

Earthworm Acute Toxicity Test

Eisenia andrei earthworms were purchased from a commercial dealer (Clover Strategy, Lda., Portugal) and set as laboratory culture maintained before starting the test. They were cultured under the same conditions and were judged to be free from contaminants. Adult earthworms, clitellated and of similar size (300-500 mg individual weight) were selected for testing. Before started the test, the earthworms were washed with deionized water and were kept on moist filter paper for a pair of hours to devoid the gut content. After that, the earthworms were placed individually in the Petri dishes. Ten replicates were used per condition, each consisting of one earthworm per Petri dish. The test was done in the dark at 22 ± 1ºC for a period of 48 h. After the exposure, earthworms were monitored for mortality.

The Table 16 shows and summarizes the results obtained in the two ecotoxicity tests for the different samples from Vigo and Ghent. The results are represented as LC_{50} (lethal concentration 50) for germination, IC_{50} (inhibitory concentration 50) for seed elongation, and LC_{50} for earthworm survival, with 95% confidence intervals.

Samples from Vigo demo plant did not show any toxic effect on seeds and earthworm. LC_{50} and IC_{50} values were higher than 100% v/v in all samples (from different monitoring campaigns). However, after BES treatments, some effects were observed in Vigo samples. The effect is possibly related to an increase in pH and electrical conductivity (EC).

The sample from Ghent did not present toxicity for the *L. Sativa* or the *E. andrei* (LC_{50} and IC_{50} > 100% v/v).

Table 16: Ecotoxicity results (germination, root elongation and earthworm survival), pH and electrical conductivity (EC) for samples received from Vigo and Ghent demo sites

Sample	Received at LEITAT	Information	GERMINATION	ROOT ELONGATION	EARTHWORM SURVIVAL	pH	EC (µS/cm)
			LC_{50} (% v/v)	IC_{50} (% v/v)	LC_{50} (% v/v)		

AnMBR permeate (AQUALIA-Vigo demo site)	04/2019	Liquid fertiliser after AnMBR	> 100	> 100	> 100	6.89	1115
Liquid Ammonium nitrate (LEITAT-Vigo demo site)	05/2019	Liquid ammonium nitrate after BES	7.90 [7.8-8.0]	1.57 [1.1-2.3]	1.89 [--]	9.26	67800
Liquid Ammonium nitrate (LEITAT-Vigo demo site)	09/2019	Liquid ammonium nitrate after BES	36.2 [34.5-37.6]	1.50 [0.9-2.4]	41.1 [35.6-42.2]	6.91	22500
AnMBR permeate (AQUALIA-Vigo demo site)	10/2020		> 100	> 100	> 100	6.93	1141
AnMBR sludge (AQUALIA-Vigo demo site)	10/2020		> 100	> 100	> 100	6.64	1274
AnMBR permeate (AQUALIA-Vigo demo site)	06/2021		> 100	> 100	> 100	7.07	1555
AnMBR sludge (AQUALIA-Vigo demo site)	06/2021		> 100	> 100	> 100	6.92	1638
AMBR permeate (Ghent demo site)	10/2021		> 100	> 100	> 100	6.89	1272

For both ecotoxicity tests performed, using seeds and earthworms, samples from Vigo demo plant (AnMBR permeate and AnMBR sludge) and Ghent demo plant (AMBR permeate) did not show any toxic effect even if undiluted.

4. Conclusions on technical integration of innovations at Run4Life demo sites

The results of MPs presence in 2020 and 2021 about black water, grey water and also in AnMBR sludge in Vigo demo site are very similar to the aforementioned literature for decentralized treatments. Outstanding the presence of Galaxolide, Tonalide, nonsteroidal anti-inflammatory drugs (such as ibuprofen) and bisphenol A, during both years. The presence of antidepressants in black water (Fluxetine and Citalopram) is more noticeable in 2021. Similarly, it shows in AnMBR sample sludge, in which polycyclic fragances and non-steroidal anti-inflammatories are the most abundant compounds detected, although MPs concentration in sewage sludge are much dependent on their physicochemical characteristics and usage rates.

NoV and SaV are most common virus thar are presence in wastewater. They are transmitted via the fecal-oral route and belong to the most infectious group of causative agents of epidemic gastroenteritis. In no case was detected this virus in reuse grey water and only reveals existence in 2 samples of black water due to sampling punctual contamination. No presence of Sars-Cov 2 found.

In order to know the pathogens content in samples and the applicability of the re-claimed water in agriculture as irrigation, grey water and black water samples were analysed. The results show that grey water meets European Regulation Framework requirements for type A quality, the highest quality required for reuse water in agriculture. Pathogen removal capacity has been less than expected but AnMBR permeate meet Type B quality requirements in 70% of samples taken which it allows all irrigation methods in agricultural use for food crops consumed raw where the edible part is produced above ground and is no in direct contact with reclaimed water processed food crops and non-food crops including crops used to feed milk or meat producing animals.

The use of AnMBR effluent from Vigo to irrigate ray grass and basil in pot experiments showed that this effluent could provide substantial amounts on nutrients to the plants. Since the fertigation water was applied according to irrigation needs large volumes of effluent was applied without observing and deleterious effects on the crop health. Also different test evaluating toxic effect on flora and fauna repeatedly proved no toxic effects from the AnMBr effluent from Vigo. To best utilise the nutrient resources in the AnMBR effluent it could be combined with initial fertilisation to enable the plant growth at early state when irrigation need is low.

5. References

References ecotox:

US Environmental Protection Agency (1996), Ecological effects test guidelines. OPPTS 850.4200. Seed germination/root elongation toxicity test. EPA 712/C-96/154. Washington, DC.

OECD (1984), Test No. 207: Earthworm, Acute Toxicity Tests, OECD Guidelines for the Testing of Chemicals, Section 2, OECD Publishing, Paris.

Tiquia, S. M., and N. F. Y. Tam (1998), Elimination of phytotoxicity during co-composting of spent pig-manure sawdust litter and pig sludge. Bioresource Technology 65, no. 1-2: 43-49.

From paper: *Is anaerobic digestion effective for the removal of organic micropollutants and biological activities from sewage sludge?*

Virus:

Calicivirus Removal in a Membrane Bioreactor Wastewater Treatment Plant. Laura C. Sima, Julien Schaeffer, Jean-Claude Le Saux, Sylvain Parnaudeau, Menachem Elimelech, Françoise S. Le Guyader. Applied and Environmental Microbiology Vol. 77, No. 15.