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EUROPEAN UNION
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WOW!
European Regional Development Fund

TECHNICAL REPORT ON OPERATION OF DEMO SCALE SELECTOR FOR LIPIDS

DEADLINE: 28/02/2022

NAME: Technical report on operation of demo scale selector for lipids (UL)

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SUBJECT: Pilot-scale results

Table of contents

List of Abbreviation

The following abbreviations are used throughout this technical report:

AST	Activated Sludge Technology
BOD	Biological Oxygen Demand
COD	Chemical Oxygen Demand
DO	Dissolved Oxygen
EU	European Union
F/M Ratio	Food to Microorganism Ratio
FAMEs	Fatty Acid Methyl Esters; Biodiesel
FOG	Fat, Oil, and Grease
GC-MS	Gas Chromatography-Mass Spectrometry
HRT	Hydraulic Retention Time
INTERREG-NWE	European Territorial Cooperation Program within North-West Europe
LCFAs	Long Chain Fatty Acids
MLSS	Mixed Liquor Suspended Solids
OMO	Oleaginous Microorganisms
PE	Population Equivalent; Unit per capita loading
PHA	Polyhydroxyalkanoate; Bioplastics
Redox	Reduction/Oxidation reactions
SRT	Sludge Retention Time; Sludge age
SV_{30}	Volume of Settled solids after 30 minutes
SVI	Sludge Volume Index
tDM	Tons of Dry Matter; Tons of total solid content
TN	Total Nitrogen
TP	Total Phosphorus
WOW	Wider business Opportunities for raw materials from Wastewater
WWTPs	Wastewater Treatment Plants

1. Introduction

Sewage contains valuable substances that can be used as raw materials for biobased products. However, to date this potential has hardly been exploited to its full potential in North-West Europe. This results in loss of valuable materials, CO_2-emmissions and less efficient use of natural resources. The Interreg North-West Europe project WOW! - Wider business Opportunities for raw materials from Waste water (sewage) - aims to develop three value chains for the recovery of carbon based elements from sewage (see **Figure 1**):

1. **The production of biodiesel**. The sewage inflow is used to cultivate *Microthrix p.* which can accumulate lipids. The lipids are extracted, processed and transformed to biodiesel.
2. **The production of bio-oil, biochar and acetic acid**. The screening material which mainly consists of cellulose material (toilet paper) is dewatered and dried. In a thermal degradation process (pyrolysis) the dried cellulose material is converted into biochar, bio-oil and acetic acid.
3. **The production of PHA (bioplastic)**. For this the primary sludge is used. In a biological process, PHA is enriched and extracted. Then the PHA is compounded and processed to an end product.

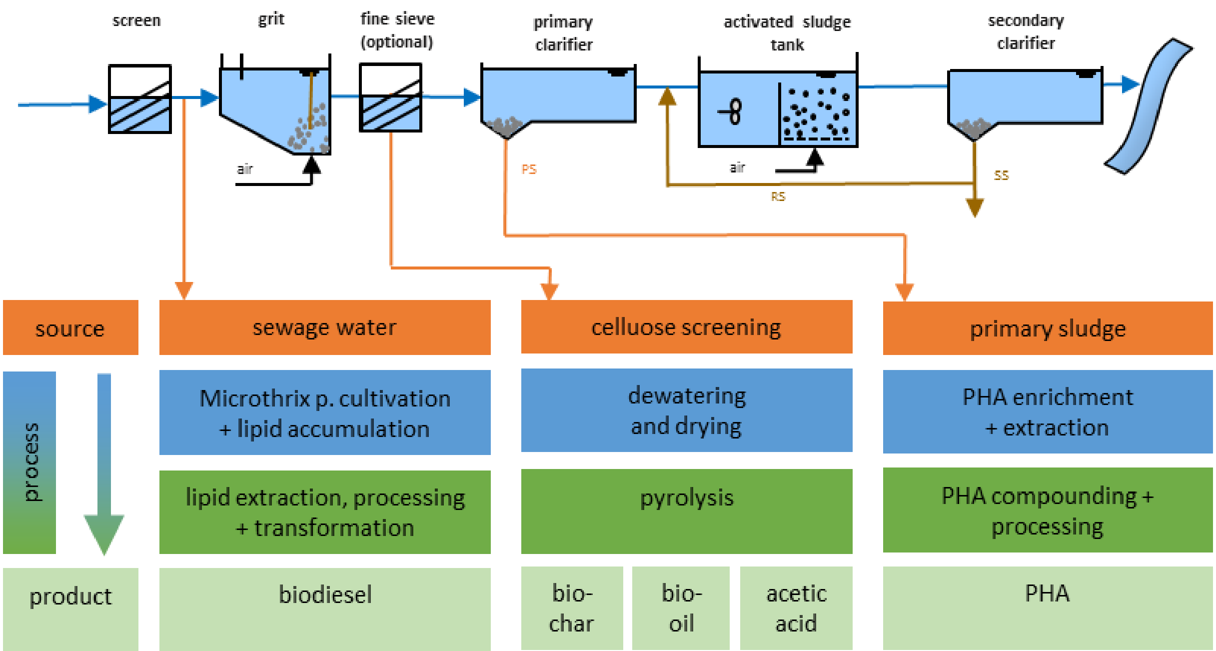

Figure 1. Recovery of carbon based elements from sewage in WoW!

One of the main activities of the project was to demonstrate the technical feasibility of these three value chains in three pilots with a focus on optimisation of the different recovery and upcycling techniques and tailoring the products to market needs.

This report focusses on the production of biodiesel from activated sludge enriched with lipids accumulating bacteria, in an up-scale Pilot Plant under "real" conditions, connected to a Wastewater Treatment Plant (WWTP), from January to September 2021 at the University of Luxembourg, within the Chair for Urban Water Management.

The lipids pilot plant was set with favorable operational parameters, based on the results of lab-scale experiments, to provide optimum growth conditions for *Microthrix parvicella (M. parvicella)*. According to the first hypothesis of this work, *M. parvicella* would be one of the most known filamentous oleaginous microorganisms (OMO) in urban wastewater treatment plant (WWTP), and it should be used to enhance lipid accumulation from inlet sewage. The main objectives of the lipids pilot plant installation were:

- Assessment of influent wastewater quality to identify the potential biodiesel production from lipids accumulation by OMO;
- Assessment of the influence of main parameters (design and operational) of the lipids-pilot plant on lipids accumulation by OMO;
- Evaluation of microbial community development at the sewage sludge, according to experimental time-duration;
- Test of the sewage sludge settleability and its recirculation in the pilot to accumulate lipids;
- Characterization of Long chain fatty acids (LCFAs) in the influent and effluent wastewater, and those accumulated in the set of bioreactors;
- Quantitative assessment of Fatty acid methyl esters (FAMEs) or Biodiesel production from lipids extraction;
- Pilot plant performance evaluation by comparing the biodiesel production from lipids accumulated to those from extracted lipids already existing in the activated sludge.
- Influence of lipid-pilot on the operation of conventional treatment plant, more specifically, the nutrient elimination.

2. Material and methods

2.1. Description of SIVOM de l'Alzette WWTP

The SIVOM de l'Alzette WWTP, where the lipids pilot was installed, has a maximum capacity of 24,500 population equivalent (PE) and it has been operating since 1997, using combined sewerage system with Activated Sludge Technology (AST) and aerobic sludge stabilization. The WWTP is located in

Audun-le-Tiche (FR), on the Luxembourgish border, and connects 5 neighboring cities (Audun-le-Tiche, Russange, Rédange, Thil and Villerupt), which accounts for c.a. 20,000 PE.

The sewage sludge production of the WWTP counted 176.7 tDM in 2019, and the whole sludge production was disposed in compliance with French directives, as composting (Eaufrance, 2019). The AST sewerage system of SIVOM de l'Alzette is composed by an activated sludge aeration basin, without a primary sludge. So, the total amount of the surplus sludge production comes from activated sludge.

For the installation of the lipids pilot-plant in the WWTP of Audun-le-Tiche, an authorization was requested to the state service ("police de l'eau") documenting process description and operation of the plant together with possible measures to be taken in order to minimize the impact of the lipids-pilot installation to the well-functioning of the WWTP.

Although the risk was considered minimum due to the small daily flow treated from the lipids pilot, complementary measures were taken to assure that filamentous bacteria would not contaminate the AST: a storage tank was installed to collect the outlet of the pilot-plant. The presence of microorganisms was regularly monitored with optical microscopy and eventually controlled by adding Chlorine before sending the water back to treatment process.

2.2. Lipids pilot plant

The lipid pilot was designed taking into account the laboratory scale experiments conducted during the first phase of the WOW project. The specifications were then presented to several suppliers and the tender was awarded to EnviroChemie GmbH (Rossdorf, DE), which adapted an existing concept to our needs and resulting in a 12 X 2.5m shipping container (**Figure 2** and **Figure 3**) and connected to the inlet raw water, just after the grid chamber of the WWTP.

Figure 2. Lipids accumulation pilot plant (outside view).

Figure 3. Lipids accumulation pilot plant (inside view).

The pilot plant consists of 5 cylindrical polyethylene tanks: 1) the initial buffer tank (4 m^3) is designed for mixing the influent wastewater composition, in order to serve the plant with an homogeneous inlet; 2) two bioreactors (4 m^3), one for lipid accumulation and one for the growth of lipid accumulating bacteria; 3) one separation unit (based on sedimentation) (3.5 m^3) and finally, 4) one pumping station (0.25 m^3) for the effluent. The pre-treated wastewater was stored in a Storage tank (40 m^3), before being discharged back into the main line of the conventional sewage process for treatment. Schematic set-up of the system and sampling plan is presented in **Figure 4**. A fixed, but adjustable water level was kept in the big tanks: at 95% of the operational volume in the mixing tank (MT) and reactors, and at 82% in the sedimentation unit (SU).

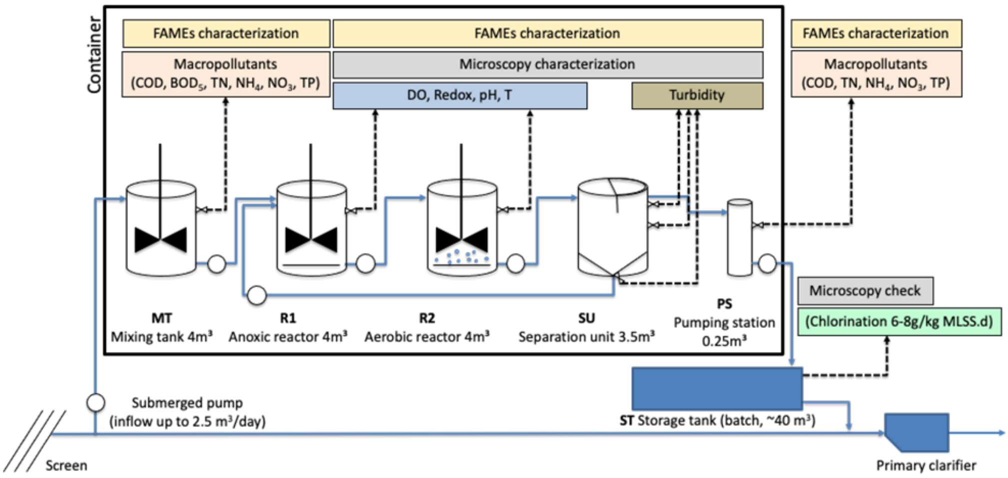

Figure 4. Schematic diagram of the pilot treatment system (Source: University of Luxembourg). The direction of the flow through the individual tanks is indicated by the solid blue lines, and sampling/sensors measurements, by the black dashed lines.

The inlet sewage was first pumped by a submerged pump protected with a coarse particle filter (Ø 2 mm) to the mixing tank (MT). Thereafter, this water was continuously pumped into the bioreactors inoculated with surplus activated sludge from AST of the WWTP. The first one, anaerobic reactor (R1), was intended for the accumulation of lipids, and second (R2), intermittently aerated reactor, was used for the growth of the lipid accumulating bacteria, according to the lesson learned from the laboratory experiment. In the first concept of the design, a challenge has been identified: the separation of solids and sludge recirculation to maintain the biomass level. Hence, a separation unit (SU) was installed with the following purposes: 1) recovery and recirculation of the lipid-rich sludge back to the reactors, 2) separation of sludge from clear supernatant; supernatant was then discharged back into the main stream line of the WWTP. Additionally, the separation unit served also as sludge storage for further dewatering, extraction and transesterification to produce biodiesel. From the SU overflow, the pre-treated water flowed to a storage tank (ST) through a pumping station (PS) where the outflow composite samples were taken.

In compliance with the national authorities requirements, the average flow into the tanks was maintained low at 2.1 $m^3.d^{-1}$ (from MT to R1), 3.4 $m^3.d^{-1}$ (to R2 and SU), and 1.5 $m^3.d^{-1}$ (recirculation of the sludge from SU to R1), which allowed a constant monitoring of the *M. parvicella* presence in the lipids-pilot' outflow. When the outside storage tank capacity (40 m^3) was reached, approximately biweekly, and before the pre-treated water being discharged back in the main line of the conventional sewage process for treatment, the presence of *M. parvicella* was evaluated by microscopic analysis, and based on the amount of solids, chlorine dose was added (6-8 g kg^{-1} MLSS d^{-1} (Övez et al., 2006; Ramírez et al., 2000)) to assure no operational problems to the hosting sewage treatment plant. However, within the operation time, it was necessary to add chlorine only twice, which confirms that the sedimentation unit worked properly, and the risk to hamper additional treatment steps was minimized.

The lipid pilot was automatically operated and regularly monitored. Both reactors were equipped with dissolved oxygen, redox potential, pH and temperature sensors to ensure the optimum conditions for *M. parvicella* growth and its lipids accumulation, as summarized in **Table 1**. The pilot was sampled two to three times per week for the evaluation of Mixed Liquor Suspended Solids (MLSS), and once per week for macropollutants (e.g. organic carbon and nutrients), Fatty Acid Methyl Esters (FAMEs) or Biodiesel, and microscopy characterization.

Table 1. Parameters and operating conditions of the Pilot plant, based on lab-scale results (WOW-Interreg, 2020).

Parameters	Values
Intermittent aeration	30-35% aerobic time slice
Microaerophilic conditions	DO < 0.5 mg/L
Sludge loads (F/M ratio)	< 0.1kg COD/kg MLSS.d
Sludge age (SRT)	> 30 days

The surplus sludge obtained from the SIVOM de l'Alzette WWTP (**Figure 5**) was used as the source of microorganisms and **inoculum** for the bioreactors R1 and R2(**Figure 6**).

Figure 5. Surplus of sewage sludge from SIVOM WWTP.

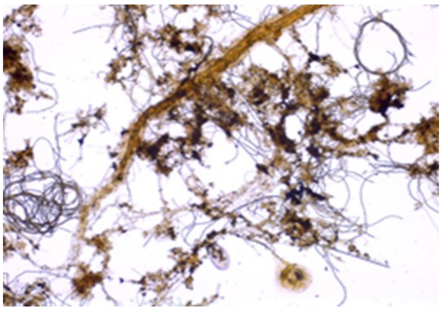

Figure 6. Inoculum with presence of M. parvicella.

The validation of the lab-scale results in a Pilot Plant under "real" conditions was performed via the monitoring of operational parameters, the general characterization of wastewater and of the bacterial community in the activated sludge.

2.3. Wastewater quality, Microbial biomass and Long chain fatty acids

The **wastewater quality** of the inflow (MT tank) and outflow (PS tank) were weekly monitored via the measurement of macropollutants parameters (COD, BOD_5, TN, NH_4^+, NO_3^-, Ortho-P and TP). The analyses were done by the University of Luxembourg, applying spectrophotometric method, based on ready-to-use bar-coded cuvette tests LCK HACH (HACH, 2020).

A general growth of **microbial biomass**, with focus on *M. parvicella* and other oleaginous microorganisms (OMO), was monitored by **Total Solids quantification, Microscopy and Genomic analyses** from the weekly sampling of activated sludge from the reactors (R1 and R2) and sedimentation unit (SU). SU was sampled in three levels, upper (SUU), middle (SUM) and bottom level (SUB). Concerning the **Solids analyses**, the Standard Methods for the examination of water and wastewater was followed (APHA, 2017).

The **microorganisms' genomic analyses** were carried out at Luxembourg Centre for Systems Biomedicine (LCSB), following their DNA-extraction and sequencing protocols for further characterization by 16S rRNA amplicon sequencing. In this case, the focus was not only on *M. parvicella*, but also on other microorganisms, that can be responsible for lipids accumulation in sewage, and which can be further converted into biodiesel. The genomic analyses allow also to verify the abundance of *M. parvicella* for the validation of microscopic monitoring protocol. **However, the genomic results will not be discussed in this report**.

The **temperature, dissolved oxygen (DO)** and **pH** parameters were monitored via automatic sensors installed inside the bioreactors to control the growth of *M. parvicella*. Most of parameters were set based on the favorable conditions for this filamentous bacterium growth (see **Table 1**), learned during the laboratory experiments. As such, the bioreactor-R2 was set to have: intermittent aeration (30-35% aerobic time interval) and microaerophilic conditions (DO < 0.5 mg/L), while bioreactor-R1 was set for anoxic condition. Even if their favorable values are known as, T: 12ºC and pH: 6.5-8.4 (Uwizeye, 2021), **temperature** and **pH** parameters, were only monitored and not controlled, as we wanted to work with the conditions present at the WWTP.

The **sludge load (F/M ratio)**, **Hydraulic Retention Time (HRT)**, both important to understand if the pilot meets the favorable conditions, and **SVI (sludge volume index, in mL/g)** were also calculated.

Because *M. parvicella* is supposed to uptake lipids like **Long Chain Fatty Acids (LCFAs)**, as a main organic carbon source to grow from the inlet wastewater (Andreasen & Nielsen, 2000; Slijkhuis, 1983), a **characterization and quantification of their composition** (i.e. C8 to C22) in influent and effluent have been carried out. Similarly, the extraction of lipids from enriched activated sludge to evaluate their accumulation has been performed together with the **transesterification into Fatty Acid Methyl Esters (FAMEs)** for the ultimately evaluation of biofuel production. Measurements have been performed by the Luxembourg Institute of Science and Technology (LIST), using Gas Chromatography-Mass Spectrometry (GC-MS).

3. Results and discussion

3.1. Monitoring of the microbial biomass

The average values of concentration of **Mixed Liquor Suspended Solids (MLSS)** found in the analyzed period were **R1: 2.94 g/L** (which is equal to kg/m3), **R2: 3.08 g/L**, **SUB: 6.63 g/L**, and **SUU: 38.83 g/L**. The average concentration value in the bottom part of the sedimentation tank (SUB) was expected to be higher than those in the bioreactors R1 and R2, as the aim of this tank is to settle and store the lipids and biomass produced in the other 2 tanks (R1: anoxic condition for lipids accumulation, and R2: aerobic condition for biomass growth).

The MLSS concentration values, together with macropollutants analyses, reactors size and the inflow wastewater rate allowed to calculate the F/M ratio, which means the quantity of food available to the microorganisms. The average values of **F/M ratio, in kg BOD5 / Kg MLSS.d**, obtained in each reactor were, **R1 and R2: 0.02**, **SUB: 0.01**, **and SUU: 0.002**, which means an ideal value of available food for *M. parvicella* growth (known as low F/M-bacterium), and consequently, lipids accumulation, in line with previous studies where, the optimum growth of *M. parvicella* was reached in low sludge loading rate (SLR) or F/M ratio (≤ 0.1 BOD_5 kg/ (MLSS kg*d), especially at low temperatures ≤ 12 to 15°C (Knoop & Kunst, 1998).

Concerning the **inflow rate**, thanks to the constant low value, around 2 m^3/d, it was possible to maintain a **high hydraulic retention time (HRT)**, which is a measure of the length of the time at which the sludge content remains in the bioreactors. The average values of HRT, within the analyzed period, in the bioreactors were: **R1 and R2: 49 hours**, and **SUB and SUU: 36 hours**, which favored a good biomass accumulation and sedimentation in the tanks versus a possible loss of biomass flushed and returning to the wastewater treatment line.

In the same context, the calculation of **SVI (sludge volume index, in mL/g)**, which means the volume (in mL) occupied by 1g of a suspension after 30 minutes settling, is used to monitor the settling characteristics of activated sludge and biological suspensions (APHA, 2017). The settleability is an important characteristic of activated sludge in biological WWTP: when poor settleability (generally caused by abundant filamentous microorganisms' growth) is observed, undesirable "bulking sludge" may occur, resulting in a loss of sludge flocs within the effluent. High SVI values, especially in low sludge loading rate, results in poor settleability due to abundant growth of filamentous microorganisms (Slijkhuis, 1983).

The monthly monitoring of SVI at the Lipids-pilot plant (**Figure 7**) shows a decrease at the end of the analyzed period due to the general increase of MLSS concentration. On the other hand, the presence of *M. parvicella* in the bioreactors was higher at the beginning of the analyzed period (see **Figure 9**), mainly because of the inoculated sludge added to the bioreactors. In the subsequent months, the growth was stabilized until started decreasing in the final of the analyzed period. As conclusion, other OMOs were favored, contributing of the MLSS increasing.

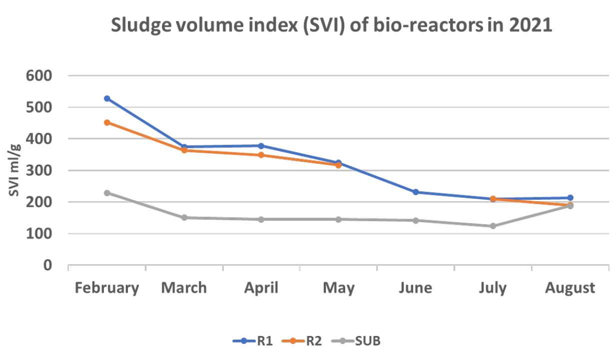

Figure 7. Sludge volume index (SVI) monitoring at the Lipids-pilot plant in 2021.

The SVI data (**Figure 7**) of the sedimentation unity (SUB) were always lower than those in bioreactors (R1 and R2). Low SVI values indicates better settleability, as from Slijkhuis (1983), as expected in a well working sedimentation unity. At the end of the analyzed period, the SVI values of Sedimentation unit (SUB) increased, mainly due to the foam formation, which appeared at the same time.

The **Filament Index** identification is used to quickly monitor the microbial community, and thus, allowed to identify the 8 most common filamentous bacteria in the lipids-pilot (descending order): *M. parvicella*, Type 1851, *Thiothrix*, Type 0675, Type 0041, Type 0092, *Sphaerotilus natans* and *Nostocoida,* as it is shown in the **Figure 8**. Although the ***M. parvicella*** **was the most representative filamentous bacteria in the bioreactors**, which was expected as the operational parameters were set to provide favorable conditions to this bacterium in line with lab-scale results, other accumulating lipids microorganisms appeared to require the same favorable conditions that *M. parvicella*.

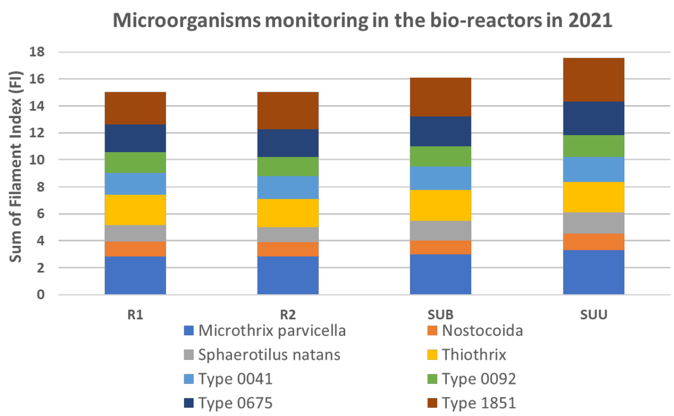

Figure 8. Microorganisms monitoring in the bioreactors at the Lipids-pilot plant in 2021.

The results of the optical microscope observation are expected to be confirmed via genomic analysis, where the relative abundance of the genera can be quantified. The genetic analyses allow also to validate the microscopic protocol to monitor the filamentous bacteria' growth by using the Filament Index.

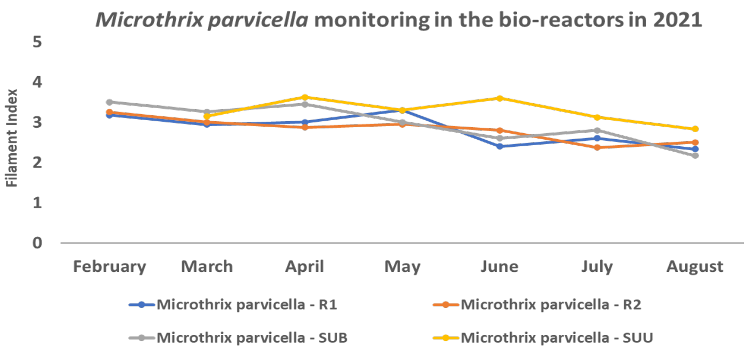

Figure 9. M. parvicella monitoring in the bioreactors at the Lipids-pilot plant in 2021.

It is possible to observe a tendency of diminishing of *M. parvicella* from May to the end of the analyzed period looking at the **Filament Index** (**Figure 9**), which can be related to the temperature increase of the summer season, which confirms the previous results from literature, where temperatures below 12^{O}C were optimal for *M. parvicella* growth (Slijkhuis, 1983; Knoop & Kunst, 1998; Uwizeye, 2021).

3.2. Characterization of lipids content and biodiesel production

The results of lipids extraction or the **lipids content**, in mg/g SS, are summarized in the **Figure 10**. The **lipids yield**, which means the amount of lipids extracted, in mg, per gram of dry solids, varied from 4% (Outlet water) to 7% (R1). In general, the lipids yield was higher in the bioreactors than the inlet and outlet wastewater, which was wished as to be accumulated in the Lipids-pilot. Compared to wastewater inflow (39.68 mg/g SS), the **bioreactors were able to accumulate, from 1.6(SUB) to 1.7(R1) times more lipids content per tank, or 56-74% more**.

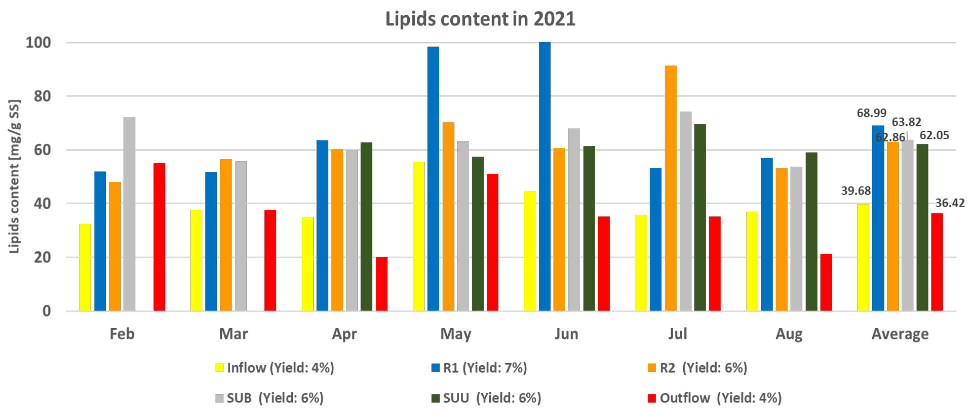

Figure 10. Lipids content at the Lipids-pilot plant in 2021.

The **transesterification process converts the extracted lipids into a valuable product that can be used to produce energy.** This product can be called as biofuel and may contain FAMEs (Biodiesel) and other types of biofuels. Therefore, the biodiesel will be characterized by the LCFA's or individual FAMEs analysis. The results of the transesterification process, summarized in the **Figure 11**, indicated an efficiency **from 70% (R1) to 80% (Inflow)**.

The performance, combustion and emission characteristics of an engine operated with biodiesel depend on the biodiesel thermo-physical properties, such as viscosity, density, cetane number, calorific value, flash and fire points, cloud and pour points, which depend upon their fatty acid contents and chemical compositions. Hence, to be used as a biodiesel in engines is mandatory to fulfil the biodiesel standard specifications required by ASTM D6751 and EN 14214 (Datta & Mandal, 2016). **Therefore, the products may need to meet standard characteristics to be used in engines.**

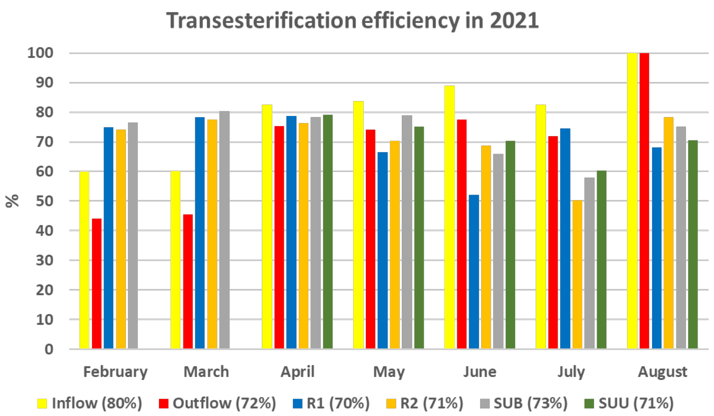

Figure 11. Transesterification efficiency at the Lipid-pilot plant in 2021.

The results of **biofuel content** (**Figure 12**), **obtained through the transesterification of extracted lipids from the inflow wastewater and the bioreactors**, were:

- the biofuel content from bioreactors was from 1.3 to 1.4 times (or 31-42%) higher (per tank) than those from lipids already existing in the inflow wastewater (32.25 mg/g).
- the sum of biofuel production from lipids accumulated in the bioreactors (R1+R2+SUB+SUU) was around, 176.86 mg/g SS, which means that the biofuel production from bioreactors was 5 times higher than from lipids already existing in the inflow wastewater (32.25 mg/g SS).
- the biofuel yield, which means the amount of biofuel produced, in mg, per gram of dry solids, varied from 2.4% (Outlet water) to 4.6% (SUB), and, the average of the bioreactors was 4.4%.

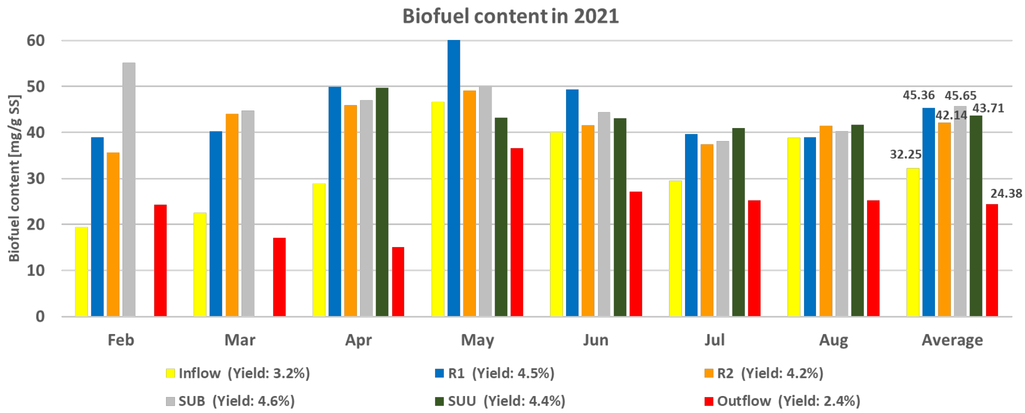

Figure 12. Total Biofuel content at the Lipid-pilot plant in 2021

Therefore, the **biofuel production due to the lipids accumulated by the pilot plant from activated sludge** (**176.86 mg/g SS**), multiplied by the sewage sludge production in 2019, which was 176.7 tDM (Eaufrance, 2019), gives an **estimated production of around 31 tons of biofuel per year**. It is important to **highlight** again that, due to the fact that **SIVOM de l'Alzette WWTP** has a **simultaneous aerobic stabilization** and therefore designed **without primary sedimentation, the total amount of sewage sludge production comes only from activated sludge**.

Concerning only **FAMEs or Biodiesel content** (**Figure 13**), **obtained through the transesterification of extracted lipids from the inflow wastewater and the bioreactors**:

- the biodiesel content from bioreactors was from 1.5 to 3.5 times higher (per tank) than those from lipids already existing in the inflow wastewater (7.31 mg/g).
- the sum of biodiesel production from lipids accumulated in the bioreactors (R1+R2+SUB+SUU) was around, 63.84 mg/g SS, which means that the biodiesel production from bioreactors was 9 times higher than from lipids already existing in the inflow wastewater (7.31 mg/g SS).
- the biodiesel yield, which means the amount of FAMEs produced, in mg, per gram of dry solids, varied from 0.3% (Outlet water) to 2.6% (SUU), and, the average of the bioreactors was 1.6%.
- overall, the biodiesel production from the bioreactors represented 36% of total biofuel production.

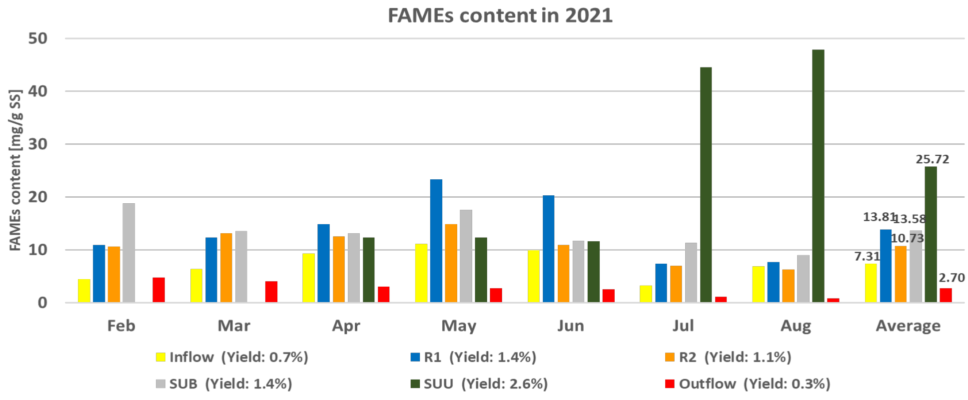

Figure 13. Biodiesel content at the Lipids-pilot plant in 2021.

Therefore, the **biodiesel production due to the lipids accumulated by the pilot plant from activated sludge** (**63.84 mg/g SS**), multiplied by the sewage sludge production in 2019, which was 176.7 tDM (Eaufrance, 2019), gives an **estimated production of around 11 tons of biodiesel per year**.

The **characterization of biodiesel components** was measured by the **individual FAMEs or LCFAs concentrations** (from C14 to C22) related to the total biodiesel produced. The biodiesel components produced from the inflow and outflow wastewater differed from those produced from bioreactors, as it is shown the **Figure 14** and **Figure 16**.

Regarding the **biodiesel components of inflow and outflow wastewater** (**Figure 14**), the contents of methyl palmitate **(C16:0)** and methyl stearate **(C18:0)** were maximal and the same, in average **39%** of total biodiesel; methyl oleate **(C18:1)** and methyl linoleate **(C18:2)** were also present, in average **18%** and **6%**, however **mostly in inflow wastewater**. According to (Jarde et al., 2005; Olkiewicz et al., 2015), the human faecal fatty acids are mostly composed by C16:0, C18:0 and C18:1, while kitchen wastes, by C16:0, C18:0, C18:1 and C18:2. Moreover, The C16-C18 lipids at high saturation level serve as good resources for biodiesel production (Zhu et al., 2017).

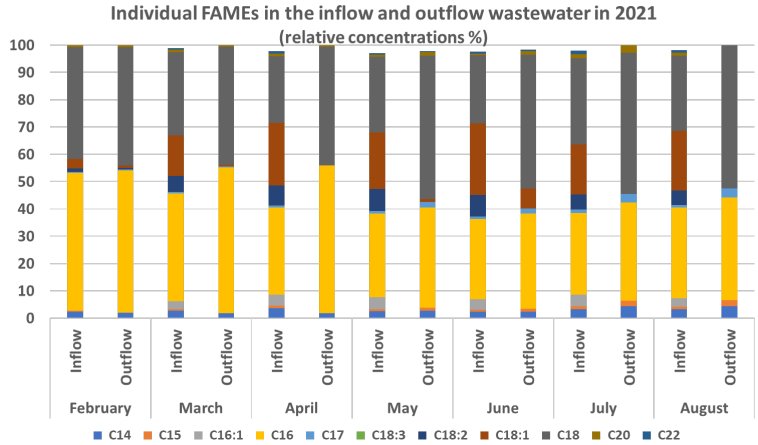

Figure 14. Individual FAMEs from inflow and outflow at the Lipids-pilot plant in 2021.

A study, comparing the biodiesel components from urban wastewaters (Frkova et al., 2020), also reported the majority presence of the fatty acids C16:0, C18:0, C18:1 and C18:2 in the inlet wastewater, as it is shown in the **Figure 15**. However, the average values of C18:1 and C18:2 were much higher compared to those from the inlet wastewater from SIVOM de l'Alzette WWTP. **Therefore, as they are among the fatty acids being accumulated in the bioreactors (Figure 16), their accumulation at the pilot plant could be even higher in other urban WWTPs with higher content of these fatty acids in the wastewater.**

The reasons for that could be in line with the literature (e.g. Dunkel et al. 2016) where a correlation, with a linear relationship, was found between the abundance of *M. parvicella* and the total LCFA loading in activated sludge (r=0.96), and linolenic acid C18:3 (r=0.98) as favorable substrate for the bacterium growth, followed by palmitoleic acid C16:1, linoleic acid C18:2, stearic acid C18:0 and palmitic acid C16:0, respectively.

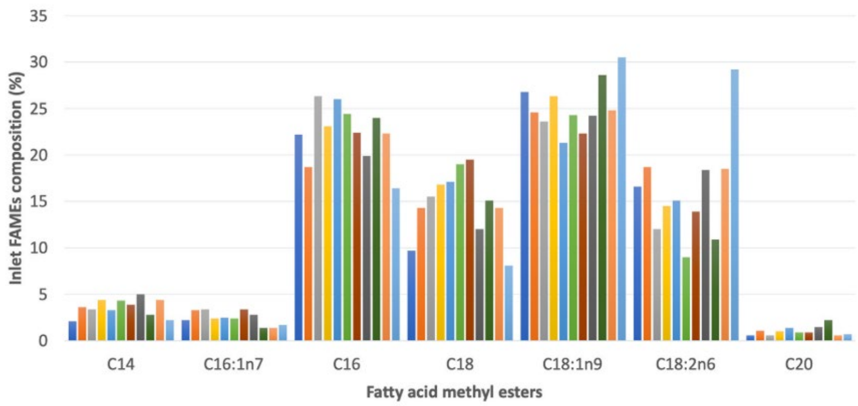

Figure 15. Concentrations of FAMEs in urban inlet wastewater (**Frkova et al., 2020**).

After analyzing the **biodiesel composition from the bioreactors Figure 16**, the content of methyl palmitate **(C16:0)** was also maximal, roughly **27%** of total biodiesel. However, as the relative concentration (%) of this fatty acid in the inlet was similar to the one in the outlet wastewater (see **Figure 14**), it can be concluded that **OMOs, including *M. parvicella*, did not use methyl palmitate (C16:0) from inlet wastewater**. Similarly, for methyl stearate **(C18:0)**, which represented 12% of total biodiesel in the bioreactors. Concerning the methyl palmitoleate **(C16:1)**, methyl oleate **(C18:1)**, and methyl linoleate **(C18:2)**, which were, 14%, 25% and 7%, respectively, they **have been found accumulated in the bioreactors**, **as their initial amount (inlet wastewater) was lower than those found in the bioreactors, and almost not present in the outlet wastewater**. C16:1 and C15:0 (which was not found in representative amount in this study) are common in secondary sludge due to the presence of microorganisms, as they considered bacterial fatty acids- part of bacteria composition (Jarde et al., 2005; Olkiewicz et al., 2015).

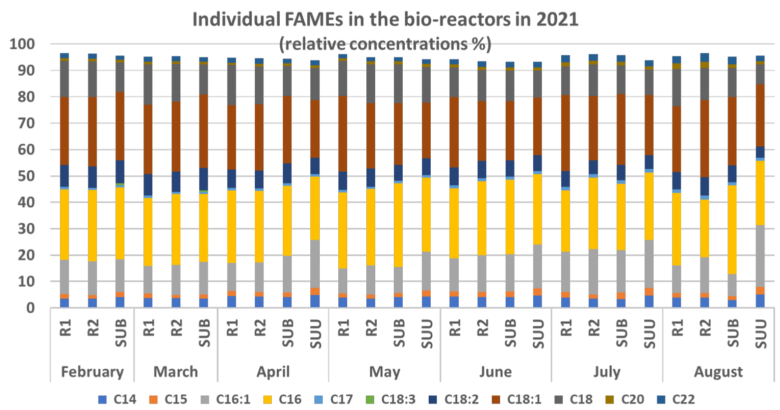

Figure 16. Individual FAMEs from the bioreactors at the Lipids-pilot plant in 2021.

To conclude, when the lipids pilot activities were ended, sludge samples of Sedimentation unity tank (SUB and SUU) were collected and sent to **ANiMOX GmbH (DE)** for **drying process** and after, the **lipids extraction and transesterification into biodiesel to assess the demo-biodiesel quality**, were done by the **National Council of Italian Research, Water research institute (CNR, IT)**. This part was supervised by **REMONDIS Aqua Industrie GmbH & Co. KG (DE)**. **The results will not be part of this report.**

3.3. Potential Biofuel and Biodiesel production at SIVOM de l'Alzette

The **potential biodiesel production of SIVOM de l'Alzette WWTP**, considering only **lipids already existing in the activated sludge**, was calculated based on data from 2019 (population equivalent of 20,000, COD load of 106 g/C.d, and the sewage sludge production from activated sludge of 176.7 tDM (Eaufrance, 2019)). Besides that, it was considered some information from literature, the lipids content in FOG from sewage sludge is 11.2 %DM (Collin et al., 2020), and the total suspended solids (TSS) removal in primary sludge and activated sludge, of 66.7% and 33.33%, respectively (Foladori et al., 2010).

The **results showed a potential biodiesel production from 0.5 ton to 6.3 tons per year from activated sludge** (considering the biodiesel yields from 0.5-6%, based on a review published on this subject by Frkova et al., 2020). The range of potential biodiesel production is due to different extraction methods.

The comparison between **biodiesel production in activated sludge**, from lipids accumulated in the pilot plant (see **chapter 3.2**) to a potential production considering those already existing in the activated sludge, allowed to analyze the **efficiency of the lipids pilot in accumulating lipids**, or in other words, to analyze the appropriateness of the operating parameters set to provide favorable conditions to OMO growth and lipids accumulation.

In that context, considering the average biodiesel yield of 1.6%, obtained in the bioreactors at the lipids-pilot, the **potential biodiesel production** of SIVOM de l'Alzette WWTP, from activated sludge (based on the lipids already existing in activated sludge) (**Table 2**), is 2 tons per year. The comparison between the **estimated annual biodiesel production from the Lipids-pilot (11 t/year-see chapter 3.2)** and the previous calculation about the **potential biodiesel production in Activated sludge (2 t/year-see Table 2)** of the SIVOM de l'Alzette WWTP, shows that **the biodiesel production, obtained through the transesterification of lipids accumulated in the pilot plant's bioreactors, was at least 5.5 higher than the biodiesel production from lipids already existing in activated sludge.**

*Table 2. Potential biofuel and biodiesel production of SIVOM de l'Alzette WWTP (*lipids already existing in activated sludge).*

Variable	Values	Units
Population connected of SIVOM-WWTP in 2019 (Eaufrance, 2019)	20,000	PE
Sewage sludge production of SIVOM-WWTP in 2019 (Eaufrance, 2019)	176.7	tDM
Lipids content in FOG from Sewage sludge (Collin et al., 2020)	11.2%	%DM
Primary sludge production (Foladori et al., 2010)	66.7%	%
Secondary sludge (Activated sludge) production (Foladori et al., 2010)	33.3%	%
Lipids content in FOG from Sewage sludge (primary sludge)-calculation	7.5%	%
Lipids content in FOG from Sewage sludge (activated sludge)-calculation	3.7%	%
Lipids yield in Activated sludge (pilot plant - average of R1, R2, SUB and SUU)	6.25%	%
Biofuel yield in Activated sludge (pilot plant - average of R1, R2, SUB and SUU)	4.4%	%
FAMES yield in Activated sludge (pilot plant - average of R1, R2, SUB and SUU)	1.6%	%
Calculation SIVOM FOG production		
Sewage sludge production per population equivalente per day	24	g/PE/day
Lipids content in sewage sludge (activated sludge)	0.9	g/PE/day
FOG content in sewage sludge (based on lipids yield of 6.25%)- activated sludge	14.4	g/PE/day
Calculation SIVOM biofuel/biodiesel production from activated sludge*		
Biofuel production in Activated sludge	0.6	g/PE/day
	5	**t/year**
Biodiesel production in Activated sludge	0.2	g/PE/day
	2	**t/year**

The same comparison for the **total biofuel production**, and considering the average biofuel yield of 4.4%, obtained in the bioreactors at the lipids-pilot, the **potential biofuel production** of SIVOM de l'Alzette WWTP, from activated sludge (based on the lipids already existing in activated sludge) (**Table 2**), is 5 tons per year. The comparison between **the estimated annual biofuel production from the Lipids-pilot (31 t/year- see chapter 3.2)** and the previous **calculation about the potential biofuel production in Activated sludge (5 t/year- see Table 2)** of the SIVOM de l'Alzette WWTP, shows that **the biofuel production, obtained through the transesterification of lipids accumulated in the pilot plant's bioreactors, was at least 6.2 times higher than the biofuel production from lipids already existing in activated sludge**.

Based on these results, it can be concluded that the lipids pilot is a promising technology to accumulate lipids from sewage sludge for downstream biofuel/ biodiesel production.

3.4. Influence of wastewater quality and other parameters in the Biodiesel production

To compare the macropollutants's concentrations of inlet wastewater to common values for municipal WWTP, the annual average inflow rate and population equivalent at the SIVOM de l'Alzette in 2019 were considered as 7,303 m^3/day and 20,000 PE, respectively, to calculate the **macropollutants loads, in g/C.d** (gram per capita and per day) of the years 2019, 2020 and 2021.

The obtained values, when compared to the specific loads of macropollutants per inhabitant (Hansen, 2020), available in the **Table 3**, showed that **BOD_5 and COD loads of SIVOM de l'Alzette were much lower than common values of municipal WWTP**, especially in 2021, where the analyzed period was only from February to August 2021, which can lead to lower amount of lipids accumulation, and therefore, lower biodiesel production, as the FOG (Fat, oil and grease) is 30–40% of COD in urban inlet wastewater (Raunkjær et al., 1994).

Table 3. Macropollutants loads of SIVOM de l'Alzette in 2019, 2020 and 2021.

Macropollutants	Specific loads per inhabitant	SIVOM de l'Alzette WWTP*		
		2019	2020	2021**
			g/C.d	
BOD_5	**60**	34	34	38
COD	**120**	106	109	65
TN	**11**	11	10	9
TP	**1.8**	1.2	1.1	1.1
COD/TN	**11**	10	11	7
COD/TP	**60**	90	100	60

* Average inflow rate: 7,303 m^3/day (2019)

* Population equivalent: 20,000 PE (2019)

** Lipids-pilot macropollutants data (from February to August 2021)

The **ratios of COD/TN and COD/TP loads in 2021** (7 and 60, respectively) were ideal regarding COD/TP, but not exactly the recommended ones for COD/TN.

Therefore, **the ratios of COD/TN and COD/TP** were supposed to interfere with the biodiesel production from bioreactors, as it is shown in the **Figure 17**, where the **peak of biodiesel production and both ratios were also in May** for the bioreactors R1, R2 and SUB. The possible reason is related to the limited nitrogen source inhibiting the division of cells and stimulated the lipid accumulation in microorganisms (Ageitos et al., 2011). Furthermore the growth-limiting factor of filamentous microorganisms is oxygen or nutrient like carbon, nitrogen, phosphorus or sulphur compound (Slijkhuis, 1983).

Besides carbon and nitrogen content, in addition to biodegradability on the production of biodiesel from sludge, the phosphorus content may also affect biodiesel production, as it was observed comparing the biodiesel production from restaurant wastewater that was higher than those from starch wastewater, due to the higher values of C/P ratio of 222.2 against 78.1 (Chi et al., 2018).

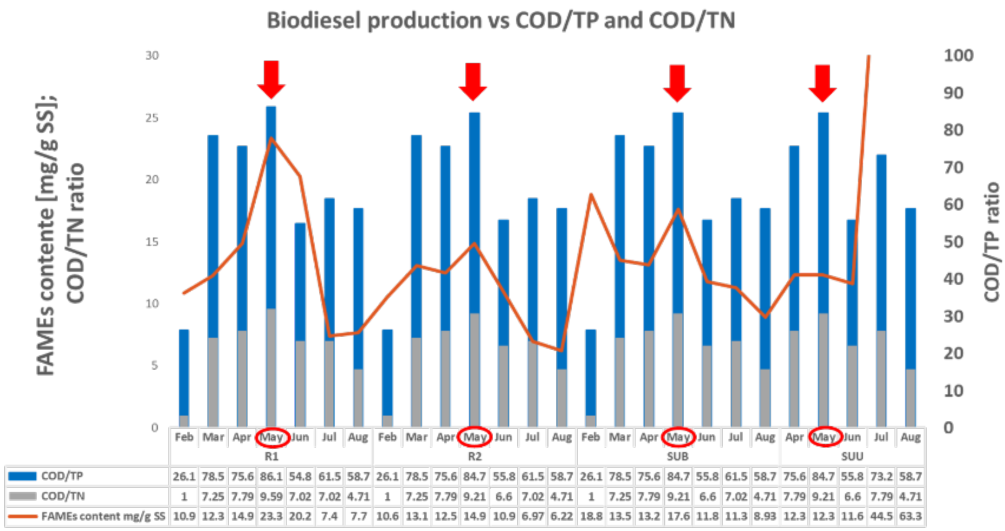

Figure 17. Comparison between Biodiesel production and COD/TN and COD/TP ratios of inlet wastewater in 2021.

To summarize, the **higher the ratio of COD/TN and COD/TP, the larger is the uptake of lipids, and consequently, the larger is the biodiesel production**, as shown in the graphs. **These values are expected to be even higher in other WWTP, with higher COD loads**.

Besides the correlation between the biodiesel production and the ratios of COD/TN and COD/TP, **the temperature also affected the biodiesel production**, due to better conditions for microorganism's growth. The comparison between the biodiesel production from bioreactors and their microorganisms monitoring (by Filament Index microscopic method), showed that the **peak of biodiesel production was in May for all bioreactors (R1, R2 and SUB)** (**Figure 18**). *M. parvicella* growth is favored at low temperatures (<12ºC), that started increasing due to the summer time.

Concerning the **SUU**, which is the top level of the sedimentation unit (SUB), the production of biodiesel was higher at the end of the analyzed period, mainly due to the **foam formation**, which is composed by sludge flocs and these filamentous microorganisms, that causes poor settleability (high SVI values), increased in the last month (see **Figure 7**).

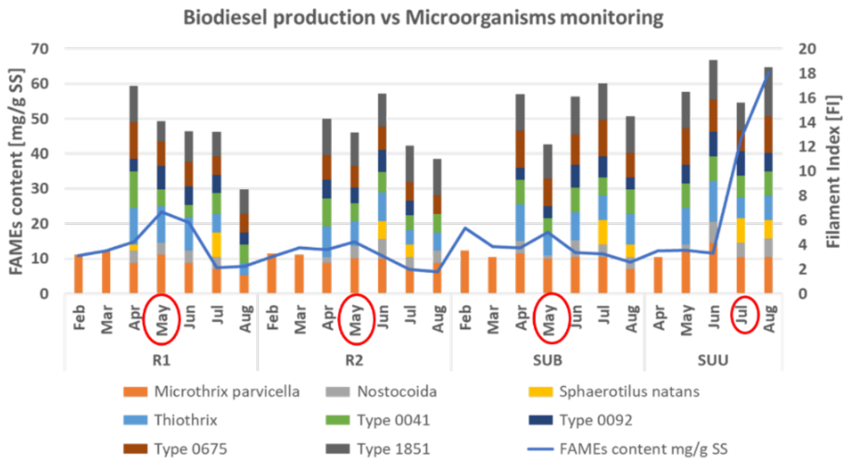

Figure 18. Comparison between Biodiesel production and Microorganisms monitoring in the bioreactors in 2021.

To conclude, **the highest biodiesel production occurred in May, when growth of *M. parvicella* is favored by medium to low temperatures**.

3.5. Challenge of the integration of a lipids pilot in conventional WWTP

The substrate depletion is one potential challenge of the lipids pilot installation in WWTPs, as the outlet wastewater from the lipids pilot plant will return to the wastewater treatment line with lower concentrations of COD, BOD, lipids and other substrates, consumed by OMO.

Modern WWTPs are based on biological treatment processes, carried out by bacteria (activated sludge), which, besides other conditions, use these pollutants from wastewater as a substrate to growth and survive (energy production). Thus, the WWTPs must be prepared to deal with this poor inlet wastewater to achieve the effluent requirements, which according to EU wastewater directives, stablished concentrations (in mg/L) of **COD** ≤125, **BOD** ≤25, **TN** ≤15 (>10.-100.000PE) and ≤10 (>100.000PE), **TP** ≤2 (>10.-100.000PE) and ≤1 (>100.000PE). The biological elimination of carbon (BOD and COD), for instance, as it is carried out by heterotrophic bacteria, besides other conditions, it requires the organic carbon for their growth (Sludge loading of ≤0.3 kg BOD_5/MLSS.d) (Hansen, 2020).

In that context, within the analyzed period in 2021, the elimination of macropollutants at the lipids pilot plant, summarized in **Table 4**, shows that the consumptions of **COD** and **BOD_5** by OMO were, respectively, **74.8** and **86.8%** of the inlet wastewater concentrations, and the final values were in accordance with the range for effluent requirement. The elimination of **TN**, was lower that the COD and BOD5, **54.6%**, but enough to achieve the required values of effluent. Concerning the elimination of **TP**, it was only **5.8%** of the inlet wastewater concentration, and the effluent value was not in accordance with the required ones.

Table 4. Macropollutants elimination by OMO in the lipids pilot plant in 2021.

Macropollutants (mg/L)	Inlet	Outlet	Elimination [%]
COD	**179.2**	**45.1**	**74.8**
BOD5*	**103.6**	**13.7**	**86.8**
NH4+	17.1	3.1	81.8
NO3-	0.5	4.7	-895.3
TN	**25.5**	**11.6**	**54.6**
Ortho-P	1.6	2.3	-44.7
TP	**3.0**	**2.8**	**5.8**

* The BOD5 of outlet was estimated by analyzing the COD-dissolved

If COD and BOD- elimination is higher than N-elimination, denitrification in the WWTP my suffer, because of the decrease of C/N-ratio. Values > 4/1 are needed; if smaller, denitrification gets hampered. Furthermore, OMO will preferably use easy degradable carbon, so this amount is reduced (also with possible negative impact on denitrification) (Hansen, 2020).

To summarize, as the main objective of WWTPs is to eliminate these macropollutants of the inlet wastewater, the lipids plant should be only operated in side-stream or with a specific percentage of the whole wastewater, as it cannot eliminate the macropollutants safely to follow the effluent requirement. **Concerning the substrate depletion caused by the lipids pilot plant, dimensioning may play a great role, which was negligible in this study because of the small pilot plant size**. However, it should be considered in future studies of commercial scale.

The reduction of **biogas production in digesters**, around 13–18% (Frkova et al., 2020), which is not the case in SIVOM de l'Alzette, has to be consider in larger WWTP with sludge digestion. This challenge was exploited in the **techno-economic assessment (TEA)**, where the **estimated production cost or the minimum selling price (MSP) of biodiesel production** from sewage sludge is 1.59€/Kg, and one of the impacts in the biodiesel production is the plant scale or wastewater inflow, as the biodiesel MSP (€/Kg) decreases as the scale increases, due to lower operating expenses per unit. The most significant impact for the economic feasibility is the recirculation flow at the pilot to maintain the biomass level, where a reduction by 50% would cause a 53% reduction in biodiesel MSP (WOW-Interreg, 2021).

3.6. Engineering recommendation for the design of the lipids pilot

A general engineering recommendation for the lipids pilot' design, from the concept to applicability, can be envisaged:

- The **bioreactors design**, with continuous flow, and parameters, which were set to offer better conditions for OMO growth and lipids accumulation, worked very well, and allowed to offer optimum conditions to the OMO.
- However, the **bioreactors dimensioning** plays a great role, and it should be considered in future studies of commercial scale. The substrate depletion (esp. for COD and BOD), caused by the lipids pilot plant, can represent problems to the hosting sewage treatment plant in the safe macropollutants elimination to follow the effluent requirements of discharge; this is especially the case for nitrate-elimination (denitrification), where a specific (high) C/N-ratio is needed. Therefore, the lipids plant should be only operated in side-stream or with a specific percentage of the whole wastewater. The inflow wastewater quality (COD and BOD_5 loads, as well as the COD/TN and COD/TP ratios), also plays an important role, as it is directly related to the OMO growth and lipids accumulation.
- Concerning the **separation unit (SU)**, although it was expected a more sophisticated system (membrane or filter belt), the simple one worked, and the SU achieved its purposes: 1) recovery and recirculation of the sludge enriched with lipids and OMO to the bioreactors, 2) the good sludge settleability allowed the separation of sludge from clear supernatant to be discharged back into the main stream line of the WWTP, and avoiding its contamination by *M. parvicella* and other OMO, following the request from the national authorities, and 4) sludge storage for further dewatering, extraction and transesterification to produce biodiesel. A **turbidity sensor** would be a nice to have: in case of floatation, another valve should open in automatic mode to release the clear water separated.
- However, **sludge recirculation**, should be optimized in future installations to reduce the biodiesel minimum selling price (MSP) to be more competitive in the market price.
- The **microbial monitoring protocol** using Filament Index confirmed being a robust method to follow the optimal growth of *M. parvicella*.
- In general, **the lipids pilot had a good performance in lipids accumulation from sewage sludge**, demonstrating being a **promising technology** for downstream biofuel/ biodiesel production. However, improvements are necessary, such as:
  - ✓ The technologies of sludge collection and pre-drying process, which is associated with high costs with transportation due to the moisture content, and required techniques to maintain the quality of further processes of lipids extraction and its transesterification into biodiesel, have to be more economically viable and practical.
  - ✓ The foam harvesting to produce biodiesel by the transesterification of extracted lipids demonstrated being an interesting alternative to reduce costs, e.g. with

transportation. Moreover, the foam production can be maximized with the inflow rate reduction; however, this has to be synchronized with the control of the settlement tank (use of turbidity sensor, e.g.). In this case, an automated mechanical foam harvesting system could be also a good improvement.

4. Conclusions and future perspectives

The Lipids-pilot plant demonstrated being a promising technology to accumulate lipids from sewage sludge, as the results of accumulated lipids extracted from the bioreactors were higher than the existing lipids extracted from the inlet wastewater of SIVOM de l'Alzette WWTP, and, consequently, higher biofuel and biodiesel production, obtained through the transesterification of lipids accumulated in the pilot plant's bioreactors, compared to those from the inlet wastewater. The results indicated that the lipids content in the bioreactors, was roughly from 56 to 74% higher than those already existing in the inlet wastewater.

The estimated annual biofuel and biodiesel production, obtained through the transesterification of lipids accumulated in the pilot plant's bioreactors (31 and 11 t/year, respectively), compared to the potential production from lipids already existing in activated sludge of the SIVOM de l'Alzette WWTP (biofuel 5 t/year, and biodiesel 2 t/year), showed that the biofuel and biodiesel production from pilot plant's bioreactors was at least 6.2 and 5.5 times higher, respectively, than those from lipids already existing in activated sludge. In addition, the inlet wastewater from SIVOM de l'Alzette WWTP has a lower COD load, specially in 2021 within the analyzed period, which implies in lower lipids content, compared to common urban wastewaters composition. Therefore, the lipids accumulation at the pilot plant, and further biofuel and biodiesel conversion could be even higher in other urban WWTPs.

The assessment of the influence of main parameters of the lipids-pilot plant in the lipids accumulation by OMO confirmed the results from lab-scale experiments and literature review, which provided favorable conditions for *M. parvicella* growth and lipids accumulation.

Challenges for the biodiesel production from sewage sludge lipids on a commercial scale, are:

- Optimization in operational process of WWTP to produce biodiesel from sewage lipids without compromising its main activity (which is to have a good efficiency in microbial wastewater treatment);
- Clear and encouraging EU policies and legal framework of use of raw material from wastewater;

- Gains in competitiveness compared to conventional diesel, such as costs reduction and efficiency improvement of technologies currently available on the market, starting with the designing of bioreactors to produce biodiesel, optimization of the recirculation flow in the pilot plant to reduce the biodiesel MSP, sludge collection and transportation, dewatering process, lipids extraction, biodiesel purification to meet the standard specifications, etc.

As future perspectives, other assessments need to be done to highlight other advantages of Biofuel production from lipids accumulated by OMO from sewage sludge, such as a Life Cycle Assessment comparison between this biofuel/ biodiesel production with conventional diesel, as well as with other biofuels, e.g. biogas, to support the decision making by WWTPs. Besides that, as it was identified the presence of other accumulating lipids microorganisms in the activated sludge, a further investigation should be performed to better understand their role in lipid accumulation and finally to increase the effectivity of the whole process.

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