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Operation of a pilot-scale lipid accumulation technology employing parameters to select *Microthrix parvicella* for biodiesel production from wastewater

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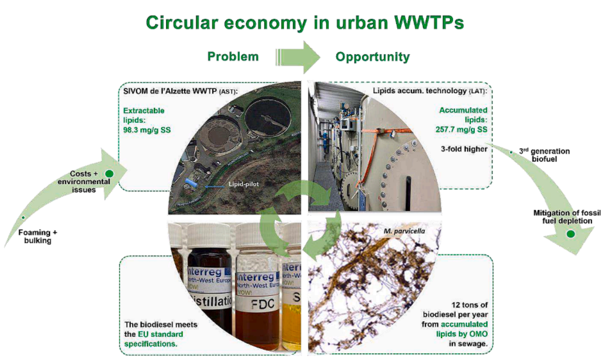
HIGHLIGHTS

- It is the very first-time that a pilot was used to accumulate lipids from sewage.
- *Microthrix parvicella* can be used to enhance the lipid accumulation in urban sewage.
- 3rd generation biofuels can be produced from accumulated lipids in urban sewage.
- Lipid-pilot is a promising technology for biodiesel production from urban sewage.

GRAPHICAL ABSTRACT



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ABSTRACT

Wastewater treatment plants (WWTPs) may play a crucial role in shifting to a zero-emission future by becoming more sustainable and contributing to the circular economy (CE). Recovered lipids from urban sewage can serve as a raw material for biofuel production contributing to a waste reduction, mitigation of natural resources depletion and reinforcing security and energy independence. A novel, pilot-scale lipid accumulation technology (LAT) employing parameters to select *M. parvicella* for the biofuel/biodiesel production was implemented on a side stream of an urban WWTP. The LAT proved its concept as the average amount of extracted lipids accumulated in the bioreactors was three-fold higher when compared to the lipids existing in activated sludge. The average transesterification of extracted lipids to biodiesel resulted in a 1.6 % yield, meaning that from 1 kg of dried sludge, 16 g of biodiesel could be formed. The biodiesel produced complies with European standard specifications (EN14214).

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1. Introduction

As a consequence of global energy market disruption, the EU proposed increasing the 2030 target for renewables to 45 % (REPowerEU-EC, 2022). Furthermore, sewage treatment may play a crucial role in shifting to a zero-emission future by becoming more resource sustainable and contributing to the circular economy (CE) (EEA, 2022). Urban sewage sludge has been studied worldwide as a potential feedstock for biofuel production as it contains an ample amount of organics including lipids (Bora et al., 2020; Liu et al., 2021). Lipids are characterized as FOG (fat, oil, and grease) with a fatty acid profile corresponding to 30–40 % of the total COD (chemical oxygen demand) in the urban sewage (Raunkjær et al., 1994).

The recovery of resources from urban sewage, as an alternative to the traditional modes of disposal via landfill and incineration, has been a subject of numerous research projects with room still for technological improvement to become less costly and sustainable (di Bitonto et al., 2016; Liu et al., 2021; EEA, 2022). Proposed solutions for lipid extraction have shown a decreasing trend in total biodiesel yield along the wastewater treatment, as lipids are readily removed by indigenous microbes. Oleaginous microorganisms (OMOs) intracellularly accumulate large amounts of lipids (>20 % of their dry weight) from an abundant carbon source and under limited nutrient conditions (Qin et al., 2017). In an assessment of biodiesel production from lipids in urban wastewater, Frkova et al. (2020) pointed out that the available literature on the use of urban wastewater as a substrate to grow OMO is limited and recent. Furthermore, in an overview of the OMOs that have been studied worldwide for this, the majority was represented by algae, microalgae, algae-bacteria, yeast, and fungi. Enhancement of both lipid accumulation and cell growth by providing optimal conditions is a bottleneck for boosting the economical aspect of biofuel production based on OMO (Bora et al., 2020; Frkova et al., 2020; El Kantar et al., 2021; Zhu et al., 2022).

Sludge with poor settling properties, resulting from an excessive growth of filamentous bacteria, is referred to as foaming and bulking in activated sludge. A thorough screening of 740 wastewater treatment plants (WWTPs) worldwide using amplicon sequencing revealed *Microthrix parvicella* as the most abundant filamentous organism in this survey (Dueholm et al., 2022). Indeed, among other filamentous bacteria in activated sludge, *M. parvicella* appeared to be the most hydrophobic adsorbing long-chain fatty acids (LCFAs) to its cells, mainly due to the extracellular lipase, present on its cells surface (Nielsen et al., 2012).

With the aim of turning a problem into an opportunity, the operation of a pilot-scale lipid accumulation technology (LAT) is demonstrated here, employing parameters to select *M. parvicella* for the biofuel/biodiesel production using urban sewage as the only feed. The main objectives of this work are: i) assessment of inlet wastewater quality for potential biodiesel production; ii) comparison of lipid accumulation by OMO in the LAT and extractable lipids existing in activated sludge; iii) assessment of lipids and biodiesel yields obtained from the LAT.

2. Material and methods

2.1. Design and operation of the pilot-scale lipid accumulation technology (LAT)

LAT was installed at SIVOM de l'Alzette WWTP, Audun-le-Tiche (FR). The WWTP (~20,000 population equivalent (PE)) operable since 1997, with a sewerage system composed of activated sludge technology (AST) and aerobic sludge stabilization, without a primary sedimentation. The overall surplus sludge production originates from AST, which amounted to 176.7 tDM in 2019, and is being disposed of via composting in compliance with French directives.

The LAT was designed and set with favorable operational parameters for *M. parvicella* growth and lipid accumulation based on the literature and lab-scale results (WOW-Interreg, 2020; Uwizeye, 2021). The pilot was constructed by EnviroChemie GmbH (Rossdorf, DE) in a 40ft shipping container and installed, just after the grid chamber, in the inlet wastewater of the WWTP. The LAT was automatically operated and regularly monitored.

The pilot-scale LAT consisted of 5 cylindrical polyethylene tanks: 1) the mix tank (4 m^3) for homogenizing the inlet wastewater, 2) a bioreactor for lipid accumulation (4 m^3), 3) a bioreactor for bacterial growth (4 m^3), 4) a sedimentation unit (3.5 m^3) and finally, 5) a pumping station (0.25 m^3) for the outlet wastewater. Fig. 1 shows a scheme of the system and sampling design. The water level of the mix tank (MT) and reactors (R1 and R2) was kept fixed at 95 % of the operational volume, and the sedimentation unit (SU) at 82 %.

A submerged pump (with a coarse particle filter of Ø 2 mm) first pumped the inlet wastewater into the mix tank (MT). Afterwards, the water was continuously pumped into the bioreactors, which were previously inoculated with activated sludge (containing *M. parvicella*). The first bioreactor (R1) was set as anoxic for the lipid accumulation. The second bioreactor (R2) was intermittently aerated (30–35 % aerobic time slice), with dissolved oxygen (DO) < 0.5 mg/L, and was intended to promote growth of the lipid accumulating bacteria. Both reactors (R1 and R2) were equipped with sensors for the monitoring of key parameters: DO, redox potential, pH and temperature. The sedimentor (SU) was intended for 1) recirculation and recovery of the lipid-rich sludge and microbial biomass, 2) settling of the sludge and separation of the overflow supernatant (outlet), and 3) storage of the sludge for further processes of sludge dewatering, lipid extraction, and its transesterification into biofuel and biodiesel. The overflowed supernatant passed through a pumping station (PS), where the outlet was sampled, and finally to a storage tank (ST) before being discharged into the mainstream line of the WWTP.

The low inlet flow (from MT to R1) was maintained at ~ 2.1 $m^3 \cdot d^{-1}$, to comply with the national requirements (Police de l'eau), and the other flows were 3.4 $m^3 \cdot d^{-1}$ (to R2 and SU) and 1.5 $m^3 \cdot d^{-1}$ (sludge recirculation from SU to R1). When the ST capacity (40 m^3) was reached, approximately 1–2 times per month, a microscopic analysis was performed on the outlet samples and a dose of chlorine was added (6–8 g · $kgMLSS^{-1} \cdot d^{-1}$) based on the amount of solids or mixed liquor suspended solids (MLSS) (Ramírez et al., 2000; Övez et al., 2006) to overcome potential problems with excessive growth of filamentous microorganisms. The outlet was then discharged to be fully treated at SIVOM. During the LAT installation, chlorine was added only twice, as the sedimentation unit worked properly, minimizing the threat of conventional wastewater treatment steps.

The pilot-scale LAT was operated from the end of January until the beginning of September 2021 in continuous flow mode. This work focuses on sampling campaigns performed between February and August. The standard deviation, presented in most of the tables and figures, quantified the fluctuation of the analyzed parameters between the sampling days (n).

2.2. Wastewater quality and microbial biomass

The time series of wastewater quality parameters, microscopic identification (once per week), as well as solid quantification (three times per week) were analyzed without technical replicates (Sefer et al., 2016). The samples were measured in duplicates/ triplicates only if their progress did not reflect well the process status or given weather conditions. The water quality parameters (chemical oxygen demand (COD), five-day measure of biological oxygen demand (BOD_5), total nitrogen (TN), ammonium (NH_4^+), nitrate (NO_3^-), *ortho*-phosphate (*ortho*-P) and total phosphorus (TP)) of the inlet (MT) and outlet (PS) were measured weekly from fresh samples using Hach Lange test cuvettes (LCK 314, 238/239, 303, 339, 348) according to the manufacturer's protocol.

To quickly evaluate the influence of the operational parameters on the evolution of microbial biomass, the bioreactors R1, R2 and SU were

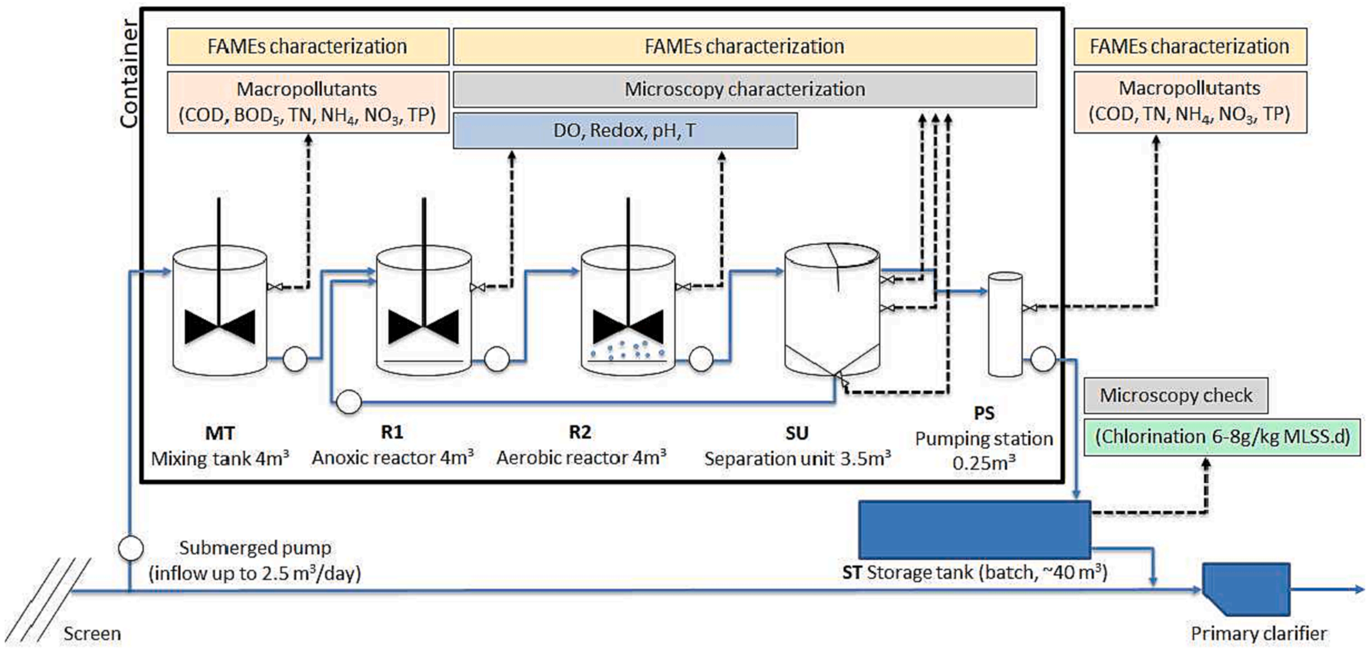


Fig. 1. Schematic diagram of the pilot-scale LAT. The flow direction through the bioreactors is indicated by the solid blue lines, and sampling or measurements are indicated by the black dashed lines.

monitored. SU was sampled in three levels, upper (SUU), middle (SUM) and bottom (SUB). These were monitored by total solid quantification: mixed liquor suspended solids (MLSS) and volatile solids (VS%), as well as sludge load (F/M ratio), hydraulic retention time (HRT) and sludge volume index (SVI), following the standard methods for wastewater examination (APHA, 2017).

The most abundant filamentous bacteria were assessed by the filament index (FI) (Eikelboom, 2000) scale from 0 to 5 (0-none, 5-very many) using Gram and Neisser staining of fixed smears by a phase contrast microscope (Leica DM1000 LED) using 20x 40x and 100x objectives.

2.3. Fatty acid methyl esters (FAMEs)

Samples for the extraction and analysis of FAMEs were taken weekly and stored at −18 °C until analyzed (one sample per month in duplicate). For liquid samples (MT, PS), an aliquot of 40 mL was sonicated (5 min 50 % 0.7cycles) while kept in an ice bath, then acidified at pH 1 with 37 % hydrochloric acid. The extraction of lipids was obtained by liquid–liquid extraction using dichloromethane (2x10mL). Organic extracts were collected, dried over anhydrous sodium sulfate, evaporated to dryness under a gentle nitrogen flow, and weighed.

For the solid samples (R1, R2, SU), the sludge was centrifuged at 4700 rpm for 10 min. The pellet obtained after the centrifugation was freeze dried for 48 h, weighed and ground prior to pressured fluid extraction (ASE 150, Thermo) with the following parameters: cell volume 1 mL, temperature 70 °C, extraction solvent mixture of *n*-hexane, acetone and methanol 6:2:2 ratio (v:v:v), and 2 static cycles. The extract was evaporated to dryness under nitrogen flow and weighed.

For the transesterification, the lipid extract was dissolved in 1 mL of *n*-hexane and transferred to a 10 mL glass tube with a Teflon-sealed screw cap, then 2 mL of freshly prepared mixture of methanol containing 1 % sulfuric acid were added. The tube was closed and incubated at 60 °C for 10 h (overnight) and allowed to cool to room temperature.

The hydrolysis was achieved by adding 2 mL of ultrapure water, then the FAMEs were recovered by liquid–liquid extraction using *n*-hexane (2x2 mL). The extract was then dried over anhydrous sodium sulfate and transferred to a pre-weighed chromatographic vial, evaporated to dryness and weighed. The obtained FAMEs extract (Biodiesel) was finally dissolved in 1 mL of *n*-hexane and diluted according to the calibration range prior to GC–MS analysis.

The quantitative analysis of FAMEs was performed using an Agilent 7890B gas chromatograph coupled with an Agilent 5977A MS detector, with a Restek Rxi-5Sil MS capillary column (30 m × 0.25 mm × 0.25 μm). Helium was used as the carrier gas (1.2 mL/min constant flow). The ionization was performed by electron impact with an electron energy of 70 eV. The injection volume was 1 μL at 280 °C and operated in splitless mode. The column temperature was maintained at 80 °C for 2 min, increased at the rate of 8 °C/min until 280 °C and then at the rate of 12 °C/min until 320 °C (12 min. isotherm). Individual FAMEs (C8, C10, C11, C12, C13, C14:1, C14, C15:1, C15, C16:1, C16, C17, C18:3, C18:2, C18:1, C18, C20, C21, C22, C23, C24) were identified by comparing their retention times and fragmentation patterns with those of the commercial standards (37 Component FAME Mix, Supelco).

3. Results and discussion

3.1. Wastewater quality and microbial monitoring

The analyses of macropollutants in the inlet wastewater were used to identify the potential biodiesel production based on its composition. The monitoring within the analyzed period is summarized in Table 1. Compared to the common values of COD/TN and COD/TP used for the AST dimensioning in urban WWTPs, ratios of 11 and 60 respectively, (DWA, 2000), the overall average ratios of the inlet that supplied the LAT in 2021 (7 and 64, respectively) were very similar to common ones for COD/TP, but much lower for COD/TN. Accordingly, the lower COD/TN ratio can lead to lower microbial growth, and consequently, to lower lipid accumulation and biodiesel yield.

Considering the average inflow rate of 7,303 $m^3 \cdot d^{-1}$ and PE of 20,000 at the WWTP case study, both 2019, the loads of BOD_5 and COD during the analyzed period (Table 1), were much lower compared to other urban WWTPs. These common values correspond to COD: 120 g $PE^{-1} \cdot d^{-1}$ and BOD: 60 g $PE^{-1} \cdot d^{-1}$ Kroiss & Klager (2018); TN: 11–13 g $PE^{-1} \cdot d^{-1}$ and 1.6–2 g $PE^{-1} \cdot d^{-1}$ Zessner & Lindtner (2005). This, can be partially explained by a higher precipitation rate that year with the

Table 1

Monthly overview of the inlet wastewater quality monitoring (biological oxygen demand-BOD, chemical oxygen demand-COD, total nitrogen-TN, total phosphorus-TP, and ratios of COD/TN-TP): average nutrient concentrations, nutrient load, and ratio of key nutrients (n = 29) ± standard deviation.

Variable	February	March	April	May	June	July	August	overall mean
				Nutrient concentration [mg/L]				
BOD_5	76.9 ± 48.7	99.7 ± 46.6	121.2 ± 37.3	116.0 ± 54.7	121.8 ± 37.4	103.0 ± 63.4	86.8 ± 82.4	**103.6 ± 48.8**
COD	89.2 ± 69.0	216.0 ± 49.7	220.8 ± 57.3	197.3 ± 81.0	207.0 ± 57.5	155.3 ± 76.4	168.7 ± 66.1	**179.2 ± 72.4**
TN	24.2 ± 13.8	32.8 ± 11.3	26.5 ± 9.0	22.5 ± 5.8	28.9 ± 6.7	19.7 ± 6.1	23.8 ± 3.5	**25.5 ± 9.0**
TP	3.2 ± 2.0	5.2 ± 4.4	2.8 ± 0.6	2.4 ± 0.7	3.3 ± 0.5	2.0 ± 0.5	2.1 ± 0.3	**3.0 ± 2.0**
				Nutrient load [g/PE.d]				
BOD_5	28.1 ± 17.8	36.4 ± 17.0	44.3 ± 13.6	42.3 ± 20.0	44.5 ± 13.6	37.6 ± 23.2	31.7 ± 30.1	**37.8 ± 17.8**
COD	32.6 ± 25.2	78.9 ± 18.1	80.6 ± 20.9	72.0 ± 29.6	75.6 ± 21.0	56.7 ± 27.9	61.6 ± 24.1	**65.4 ± 26.4**
TN	8.8 ± 5.0	12.0 ± 4.1	9.7 ± 3.3	8.2 ± 2.1	10.5 ± 2.4	7.2 ± 2.2	8.7 ± 1.3	**9.3 ± 3.3**
TP	1.2 ± 0.7	1.9 ± 1.6	1.0 ± 0.2	0.9 ± 0.3	1.2 ± 0.2	0.7 ± 0.2	0.8 ± 0.1	**1.1 ± 0.7**
				Ratio				
COD/N	3.7 ± 3.9	6.6 ± 2.0	8.3 ± 1.2	8.8 ± 1.4	7.2 ± 1.4	7.9 ± 1.4	7.1 ± 3.2	**7.1 ± 2.3**
COD/P	28.2 ± 33.5	41.2 ± 26.5	79.3 ± 5.9	80.8 ± 10.4	63.0 ± 11.9	76.3 ± 17.8	80.6 ± 25.6	**64.2 ± 23.9**

activation of combined sewer overflows, leading to discharge of loads directly into receiving waters, and a spring infiltration into the sewerage system (personal communication with the WWTP operator). Therefore, as the urban inlet wastewater COD usually consists of 30–40 % of FOG (Raunkjær et al., 1994), a lower accumulation of lipids was expected.

The ratio BOD/MLSS (F/M ratio) needs to be maintained at an appropriate level for the technology to perform well. Besides these parameters, the temperature and hydraulic retention time were monitored in the LAT and the results are summarized in the Table 2.

The overall average values of MLSS (Table 2) gradually increased between the bioreactors: R1 < R2 < SUB < SUU by 5 %, 120 % and 441 %, respectively, which was expected, as the sedimentation tank was meant to store sludge for further processes of lipid conversion into biodiesel. The average values of VS%, or the content of biomass (microorganism and organic content) in the total MLSS, show that the percentage of biomass was higher in the bioreactors R1 and R2 (79.9 and 84.88 % respectively), compared to the sedimentation unit (SUB and SUU levels, 79.2 and 78.1 % respectively), as these bioreactors were set to grow *M. parvicella* and accumulate lipids.

Concerning the F/M ratio, the overall average values, in $kgBOD_5 \cdot kgMLSS^{-1} \cdot d^{-1}$, obtained in the bioreactors were 0.02 for R1 and R2, 0.01 for SUB, and 0.003 for SUU, respectively. According to previous studies, *M. parvicella* growth was favored at low F/M ratio (≤0.1 $kgBOD_5 \cdot kgMLSS^{-1} \cdot d^{-1}$) (Slijkhuis, 1983; Knoop & Kunst, 1998; Uwizeye, 2021), which confirms that LAT offered optimum conditions to this bacterium.

A constant low flow rate of the inlet (~2 $m^3 \cdot d^{-1}$) allowed the maintenance of a high HRT in the bioreactors, 45 h for the R1&2, 35 and 34 h for SUB and SUU respectively (Table 2). HRT > 18 h were reported to favor *M. parvicella* growth (Uwizeye, 2021) and recommended for selection of this microorganism. The settled biomass in SUB was recirculated back to the R1 at 1.5 $m^3 \cdot d^{-1}$ flow rate. After 4 weeks of operation, a thick foam layer on top of the sedimentation unit was constantly being formed (SUU) and retained by a lamella separator, while a clarified transparent water overflow flowed into the pumping station and further to a storage tank, where a general check for MLSS and presence of *M. parvicella* was routinely conducted before this outlet wastewater

Table 2

Monthly overview of the LAT operational parameters ± standard deviation: temperature (n = 65), mixed liquor suspended solids (MLSS, n = 72- R1 and R2, n = 36- SUB, and n = 28- SUU), volatile solids (vS n = 10), food to microorganism ratio (F/M, n = 72- R1 and R2, n = 36- SUB, and n = 28- SUU) and hydraulic retention time (HRT, n = 65).

Parameters	February	March	April	May	June	July	August	overall mean
					R1			
Temperature (°C)	9.1 ± 3.4	10.8 ± 1.3	11.4 ± 1.7	12.9 ± 1.8	19.6 ± 1.7	19.6 ± 0.6	18.9 ± 0.7	**14.6 ± 4.4**
MLSS (g/L)	2.2 ± 0.9	2.5 ± 1	2.9 ± 0.3	3.1 ± 0.3	3.5 ± 0.3	3.8 ± 1	2.5 ± 1	**3.0 ± 0.9**
vS (%)	–	–	–	–	97.3 ± 19.5	79 ± 3.5	77 ± 0.7	**79.9 ± 4.5**
F/M ($kgBOD_5$. $kgMLSS^{-1}.d^{-1}$)	0.017 ± 0.019	0.043 ± 0.081	0.026 ± 0.007	0.023 ± 0.011	0.019 ± 0.006	0.016 ± 0.01	0.022 ± 0.027	**0.020 ± 0.013**
HRT (hours)	45.2 ± 14.2	47.9 ± 14.4	41.5 ± 0.3	41.8 ± 0.5	43.1 ± 1.8	72.4 ± 45.4	50.4 ± 4.4	**45.0 ± 11.5**
					R2			
Temperature (°C)	9.1 ± 3.5	11.1 ± 1.3	11.6 ± 1.6	13.1 ± 1.8	20.2 ± 1.7	19.9 ± 0.5	19.1 ± 0.4	**14.9 ± 4.5**
MLSS (g/L)	2.6 ± 0.7	2.7 ± 1.2	3 ± 0.3	3.2 ± 0.2	3.6 ± 0.3	3.8 ± 0.7	2.7 ± 1.2	**3.1 ± 0.8**
vS (%)	–	–	–	–	97.5 ± 16.3	81.2 ± 8.7	76.8 ± 0.8	**84.8 ± 6.1**
F/M ($kgBOD_5$. $kgMLSS^{-1}.d^{-1}$)	0.013 ± 0.015	0.045 ± 0.099	0.025 ± 0.007	0.022 ± 0.01	0.018 ± 0.005	0.016 ± 0.008	0.02 ± 0.024	**0.020 ± 0.010**
HRT (hours)	45.5 ± 14.5	47.8 ± 14.4	41.5 ± 0.3	41.7 ± 0.5	43.1 ± 1.8	72.3 ± 45.4	50.5 ± 4.3	**45.0 ± 10.3**
					SUB			
MLSS (g/L)	4.5 ± 0.5	6.6 ± 0.5	7.5 ± 2.7	7.1 ± 1.2	7.2 ± 0.9	8.2 ± 0.9	5.4 ± 1.1	**6.8 ± 1.6**
vS (%)	–	–	–	–	85.5 ± 5.2	77.2 ± 1.1	75.7 ± 0.3	**79.2 ± 5.1**
F/M ($kgBOD_5$. $kgMLSS^{-1}.d^{-1}$)	0.009 ± 0.008	0.01 ± 0.005	0.014 ± 0.007	0.013 ± 0.008	0.014 ± 0.004	0.008 ± 0.001	0.012 ± 0.014	**0.011 ± 0.006**
HRT (hours)	38.5 ± 18.2	34.5 ± 1.9	32.2 ± 0.3	32.4 ± 0.6	31.9 ± 0.9	42.4 ± 17.5	38.2 ± 3.4	**35.0 ± 9.5**
					SUU			
MLSS (g/L)	–	36.2 ± 2	31.4 ± 5.8	36.5 ± 5.9	31.8 ± 15.8	45.6 ± 12.4	51.4 ± 18.6	**36.9 ± 12.6**
vS (%)	–	–	–	–	81.1 ± 1.6	77.5 ± 0.7	75.7 ± 0.4	**78.1 ± 2.3**
F/M ($kgBOD_5$. $kgMLSS^{-1}.d^{-1}$)	–	0.002 ± 0.001	0.003 ± 0.001	0.002 ± 0.001	0.004 ± 0.003	0.002 ± 0.001	0.001 ± 0.001	**0.003 ± 0.002**
HRT (hours)	–	33.9 ± 0.1	32.2 ± 0.3	32.4 ± 0.6	31.9 ± 0.9	42.4 ± 17.5	38.2 ± 3.4	**34.2 ± 8.8**

was discharged to be fully treated by the WWTP.

The microscopic sludge investigation in the LAT, by the filament index (FI), besides having facilitated a quick correction of the treatment process (when necessary) (Eikelboom, 2000), identified the 8 most encountered filamentous bacteria: *M. parvicella*, Type 1851, *Thiothrix*, Type 0675, Type 0041, Type 0092, *Sphaerotilus natans* and *Nostocoida limicola,* in descending order (Fig. 2). *M. parvicella* was the most abundant filamentous bacterium, which was expected as the LAT was operated providing the optimum conditions to this bacterium. However, a phylogenetic study of the overall community of OMOs would be highly recommended, as it is already known that there is a plenty of them naturally occurring in activated sludge (Muller et al., 2014). Such information may further help to adjust OMO growth parameters and optimize the lipid accumulation.

A clear decreasing pattern in *M. parvicella* abundance was observed in all bioreactors from May/June (Fig. 2). Such decline could be linked to the high summer temperatures, which is further supported by previous findings, where temperatures ≤ 12 °C favored the growth of *M. parvicella* (Slijkhuis, 1983; Knoop & Kunst, 1998).

The SVI characterizes the sludge settleability in biological WWTP. High SVI values indicate weak sludge settleability given by the excessive growth of filamentous microorganisms (Slijkhuis, 1983), resulting in undesirable bulking sludge and deteriorated quality of the outlet wastewater. As observed in Table 3, the SVI monitoring of the SUB (with 123.6 to 228.6 mL/g average range) presented lower values compared to the bioreactors R1 and R2 (213.2 to 528.3, and 189.9 to 451.5 mL/g, respectively), which indicates better settleability, as expected of the sedimentation unit's ability to retain the microbial biomass and separate it from the outlet wastewater; this was one of the main objectives of the LAT.

Table 3
Monthly overview of the average sludge (SVI) in the individual bioreactors ± standard deviation (n = 36- R1 and R2, n = 32– SUB, and n = 28- SUU).

Month	SVI monitoring - LAT Bioreactors [ml/g] Mean (±Std. Dev.)		
	R1	R2	SUB
February	528.3 (±149.3)	451.5 (±268.0)	228.6 (±6.8)
March	374.4 (±52.3)	363.3 (±50.0)	150.1 (±13.2)
April	377.1 (±11.5)	348.6 (±13.0)	144.4 (±43.4)
May	323.8 (±38.3)	316.5 (±20.5)	144.4 (±24.8)
June	276.9 (±20.0)	276.4 (±14.9)	140.7 (±17.5)
July	278.7 (±53.2)	279.1 (±32.0)	123.6 (±14.2)
August	213.2 (±43.9)	189.9 (±43.7)	187.8 (±38.0)

The monitoring of SVI at the LAT pilot (Table 3) revealed a decline towards the end of the analyzed period, mainly as a result of the general ascent in MLSS concentration. In compliance with the decreasing SVI, the microscopy monitoring of *M. parvicella*, showed higher abundance at the beginning of the operational period (see Fig. 2), as the sludge which served as inoculum was already rich in *M. parvicella*. Until May (temperature ≤ 12 °C), the observed abundance was about constant with the subsequent decrease during the summer months. Elevated temperature

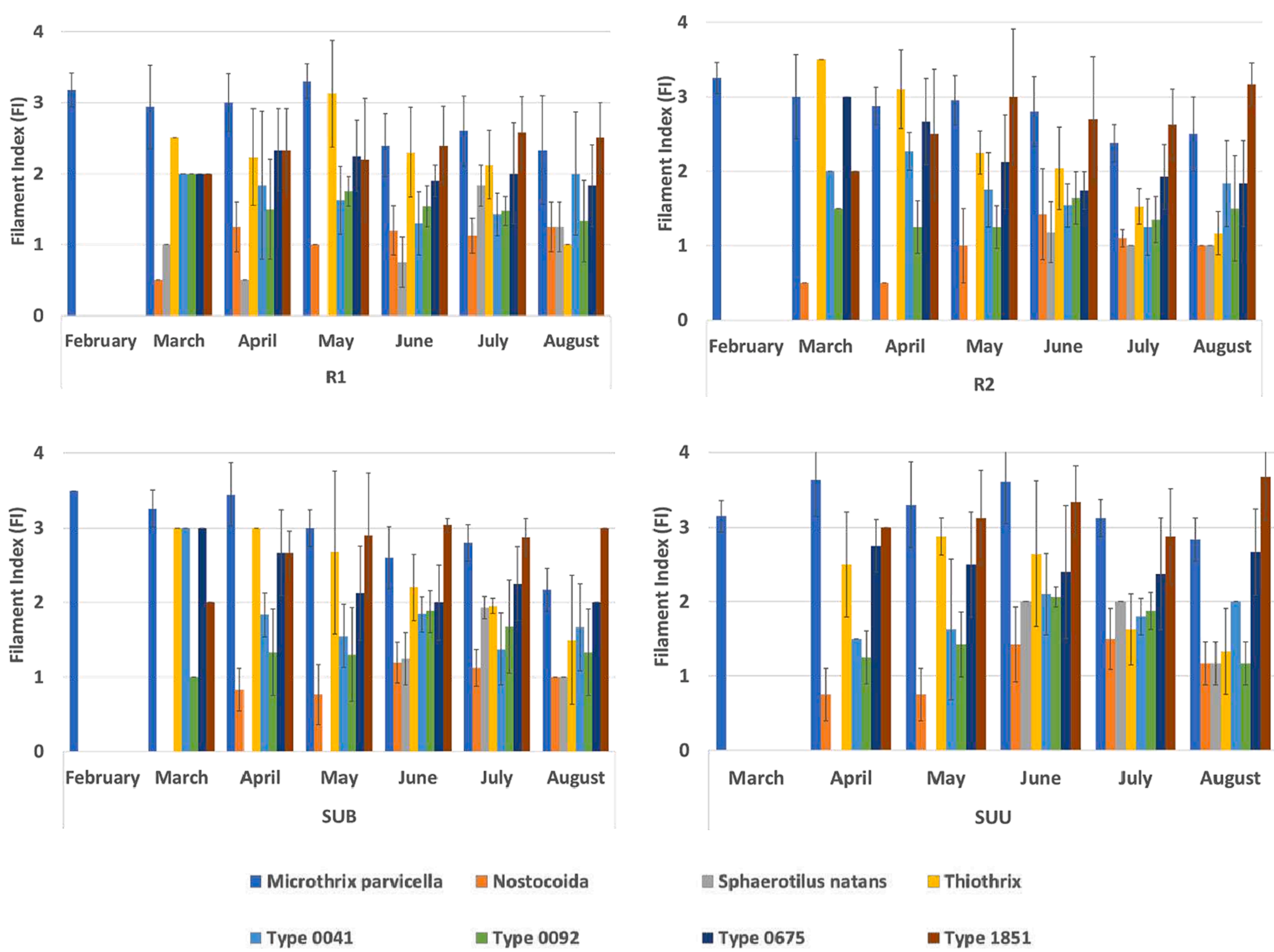


Fig. 2. Microscopic sludge monitoring: monthly average filament indexes of the most abundant filamentous bacteria present in the individual bioreactors within the entire operational period (n = 29 per each bioreactor and depth). Error bars represent standard deviation.

promoted growth of other OMOs, which took over and contributed to the increasing MLSS concentration.

3.2. Content of lipids and production of biofuel and biodiesel

3.2.1. Content of lipids

The average amount of extracted lipids, in mg/g SS (SS: suspended solids or sludge dry matter), including the lipid yield (in %), are shown in Fig. 3. In the inlet wastewater, the content of lipids was on average 39.7 mg/g SS. Up to a 1.7-fold increase in lipid content was observed in the individual bioreactors, ranging between 62.0 and 68.9 mg/g SS. Based on the average lipid yield of the bioreactors (6.25 %), the average amount of lipids accumulated by the OMOs in the bioreactors was 257.7 mg/g SS (R1 + R2 + SUB + SUU).

The estimated lipid amount, which could be potentially extracted from an AST (without the LAT installation), ranges from 32.1 to 196.2 mg/g SS, with an average of 98.3 mg/g SS. This was based on a study (Jardé et al., 2005) on the lipid fraction of sewage sludge in French urban WWTPs (with capacity from 3,375 to 24,500 PE). Therefore, returning to the estimated average amount of lipids accumulated by the OMOs in the bioreactors of 257.7 mg/g SS, the accumulation of lipids in the LAT was around three-fold higher compared to the extractable amount from the overall activated sludge, and consequently, a higher amount of biodiesel could be produced.

Considering the production of sewage sludge at the WWTP in 2019 (176.7 tDM), which comes only from the activated sludge, the estimated accumulation of lipids in bioreactors could be around 45.5 tons per year or the lipid load of $6 g_{dried\ lipids} \cdot PE^{-1} \cdot d^{-1}$. This estimation was based on the PE of 20,000 and 365 days a year.

After the lipid extraction, the next step is their conversion into biodiesel, which is the envisaged final product of this work. The esterification/transesterification are considered the most suitable method for this when sewage sludge is used as feedstock, in which FAME (or biodiesel) is formed from fatty acids and triglycerides present in sewage sludge. Besides biodiesel, other by-products could be also valorized, e.g., the intermediate fluid after methanol recovering presents a high thermal stability and is suitable for methane production (Liu et al., 2021).

As this intermediate fluid could be used as a renewable energy source, it was named here as a biofuel. The transesterification efficiency of extracted lipids into biofuel was from 70 to 73 %, and biodiesel comprised on average 37.9 % of total biofuel.

3.2.2. Production of biofuel and biodiesel

The overall average biofuel production from lipids accumulated in the LAT was 176.7 mg/g SS. This is separated as follows: R1 (45.4 mg/g SS) + R2 (42.1) + SUB (45.7) + SUU (43.5), with 4.4 % as average biofuel yield (Fig. 4). Considering the sewage sludge production at the WWTP case study in 2019 (176.7 tDM), the estimated annual production is around 31 tons of biofuel. Focus on biofuel composition was not within the scope of this work, and it will not be discussed further.

Regarding the biodiesel production, 66.9 mg/g SS was obtained from LAT's bioreactors (Fig. 4). Around 12 tons of biodiesel per year could be obtained from the annual sewage production of the WWTP case study, based on data from 2019. Overall, the average biodiesel yield obtained from the LAT was 1.6 %, which means that 16 g of biodiesel could be produced from 1 kg of dried sludge. The quality of the produced biodiesel is compliant with the European standard specification (EN14214) (Arimi et al., 2022).

Inlet wastewater composed of high organic content, along with high COD/TN and COD/TP, led to a higher accumulation of lipids, and consequently, to a possible higher biodiesel production, which was the result of a case study with restaurant wastewater (C/P ratio of 222.2) compared to starch wastewater (C/P ratio of 78.1) (Chi et al., 2018). In the LAT influent, the highest ratios of both COD/TN and COD/TP (Table 1) concurrently with a peak in biodiesel production in the R1 and R2 (Fig. 4) was observed in May. It was likely attributed to the nitrogen limitation and related cell division inhibition which stimulates the accumulation of lipids by microorganisms (Ageitos et al., 2011). In stressful conditions, such as nutrient limitation (like limited nitrogen or high C/N ratio), the carbon in the wastewater is accumulated by microorganisms in lipid form (Cea et al., 2015).

As anticipated, the temperature also had an influence on the biodiesel production due to stimulation of microbial growth. *M. parvicella* growth in activated sludge is favored at low temperatures (<12 °C to 15 °C) and its multiplication hinders when T ≥ 20 °C (Knoop & Kunst, 1998). Based on the monitoring of *M. parvicella* abundance (Fig. 2) and the content of biodiesel in the individual bioreactors (Fig. 4), the highest biodiesel production was observed in May for the bioreactors R1 and R2, with a subsequent decrease in the next month. The same trend was observed for the *M. parvicella* abundance, which declined with the

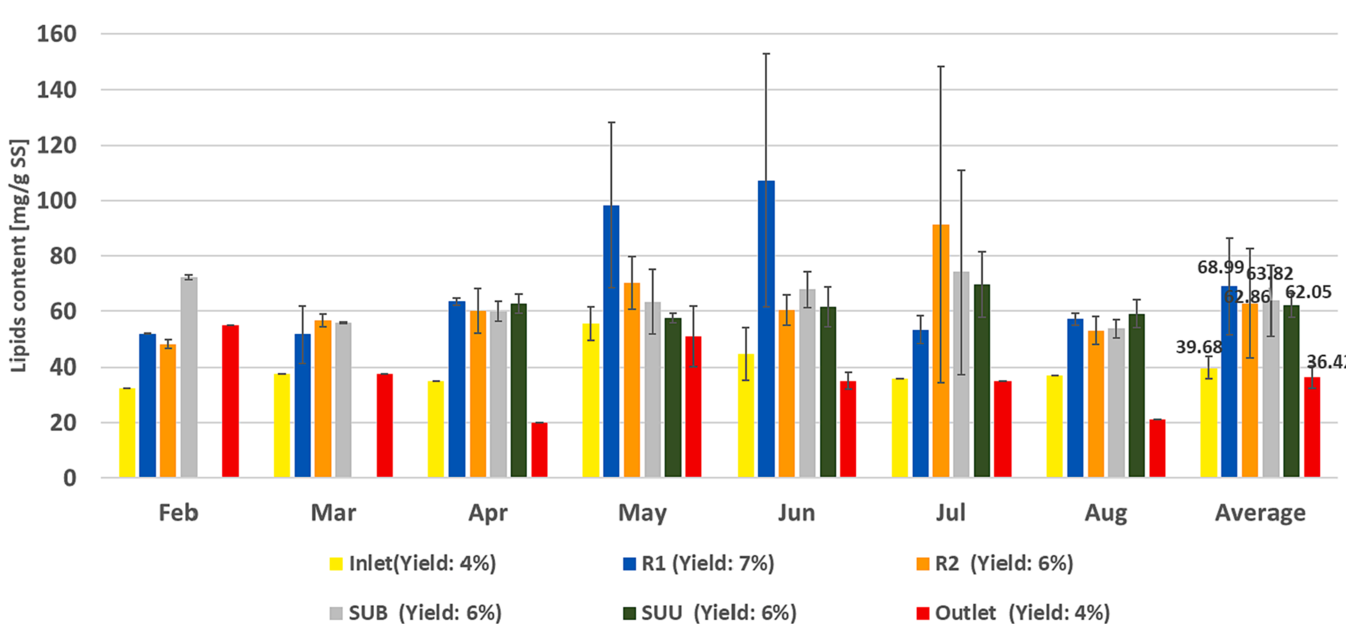
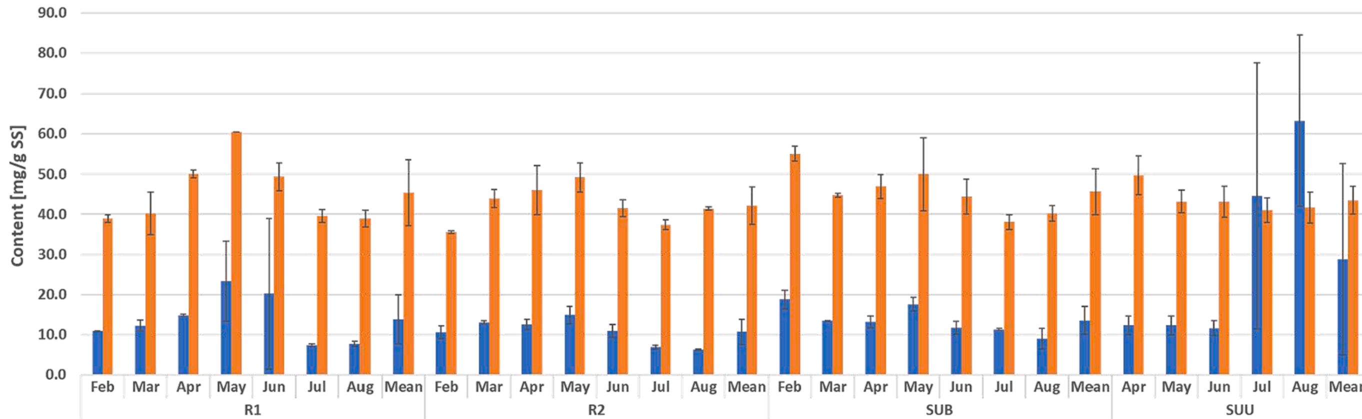


Fig. 3. Average lipids content in the inlet (n = 9), outlet (n = 9), and bioreactors R1 (n = 17), R2 (n = 15), sedimentor bottom (SUB, n = 15) and upper level (SUU, n = 13) illustrated for the entire operational period. Error bars represent standard deviation.



R1	Feb	Mar	Apr	May	Jun	Jul	Aug	Mean
FAMEs	10.9	12.3	14.9	23.3	20.2	7.4	7.7	13.8
Biofuel	38.9	40.2	50.0	60.5	49.3	39.6	38.9	45.4

R2	Feb	Mar	Apr	May	Jun	Jul	Aug	Mean
FAMEs	10.6	13.1	12.5	14.9	10.9	7.0	6.2	10.7
Biofuel	35.6	44.0	45.9	49.2	41.5	37.4	41.4	42.1

SUB	Feb	Mar	Apr	May	Jun	Jul	Aug	Mean
FAMEs	18.8	13.5	13.2	17.6	11.8	11.3	8.9	13.6
Biofuel	55.1	44.7	47.0	50.0	44.4	38.1	40.2	45.7

SUU	Apr	May	Jun	Jul	Aug	Mean
FAMEs	12.3	12.3	11.6	44.5	63.3	28.8
Biofuel	49.7	43.2	43.1	41.0	41.6	43.5

Fig. 4. Course of mean biofuel and biodiesel content in the individual bioreactors throughout the operational period. R1 (n = 17), R2 (n = 15), SUB (n = 15) and SUU (n = 13). Error bars represent standard deviation.

arrival of high summer temperatures. The opposite was observed in the SUU; biodiesel production rose in the last months of the operation period, presumably due to the increased formation of foam, rich in microbial cells.

3.2.3. *Fames composition*

FAMEs of inlet and outlet wastewater, detailed in Fig. 5, were mostly composed of C16:0 and C18:0 (~39 %), with subsequent C18:1 and C18:2 (18 and 6 % respectively), which were present mostly in the inlet. This is in line with previous observations (Jardé et al. (2005); Olkiewicz et al. (2015)), as kitchen waste is mainly composed of C16:0, C18:0, C18:1 and C18:2, while feces are of C16:0, C18:0 and C18:1.

Frkova et al. (2020) reported these four methyl esters being usually found in inlet wastewater by assessing the biodiesel from urban wastewaters, with average concentrations of C18:1 and C18:2 substantially exceeding the values encountered in this work. As these methyl esters were among those accumulated most in the bioreactors (Fig. 5), we may assume that their accumulation could be much higher when supplied with another urban WWTP inlet richer in COD and thus FOG.

This assumption can be confirmed by the study of Chipasa & Mędrzycka (2008), where they noticed variation of individual fatty acid content during the microbial growth phases, showing that LCFAs are used as a food by OMO. Dunkel et al. (2016) found that the abundance of *M. parvicella* is linear correlated (r = 0.96) to the load of LCFA in activated sludge, with C18:3 as a favorable substrate, followed by C16:1, C18:2, C18:0 and C16:0. The same was observed in a lab-scale study, where the *M. parvicella* abundance was associated with C12:0, C18:1, C18:2 and C20:0 (WOW-Interreg, 2020).

The FAMEs composition within the bioreactors did not differ much (Fig. 5), and the most abundant esters were methyl palmitate (C16:0, ~27 %) and oleate (C18:1, ~25 %), followed by methyl palmitoleate (C16:1, ~14 %), stearate (C18:0, ~12 %) and linoleate (C18:2, ~7%). According to Yi et al. (2016); Chi et al. (2018), C16:0 and C18:1 are common in activated sludge regardless of the type of wastewater. AST contains an abundance of cellular lipids (>54 %), composed of C16-C18, which are suitable for biodiesel production at high saturation level (Zhu et al., 2017). High levels of C16:1 in AST are associated with the presence of microorganisms, as it is a constituent of their membrane phospholipids (Jardé et al., 2005; Olkiewicz et al., 2015). This explains the higher level of C16:1 in the SUU compared to the other bioreactors (Fig. 5), which can be related to a thick foam layer due to the growth of OMOs.

3.3. *Challenge of the LAT integration at conventional WWTPs*

One of the drawbacks to consider- as it is one of the ways to reduce costs and environmental impacts- when integrating a LAT at conventional WWTPs, is the decrease in biogas production, as part of the

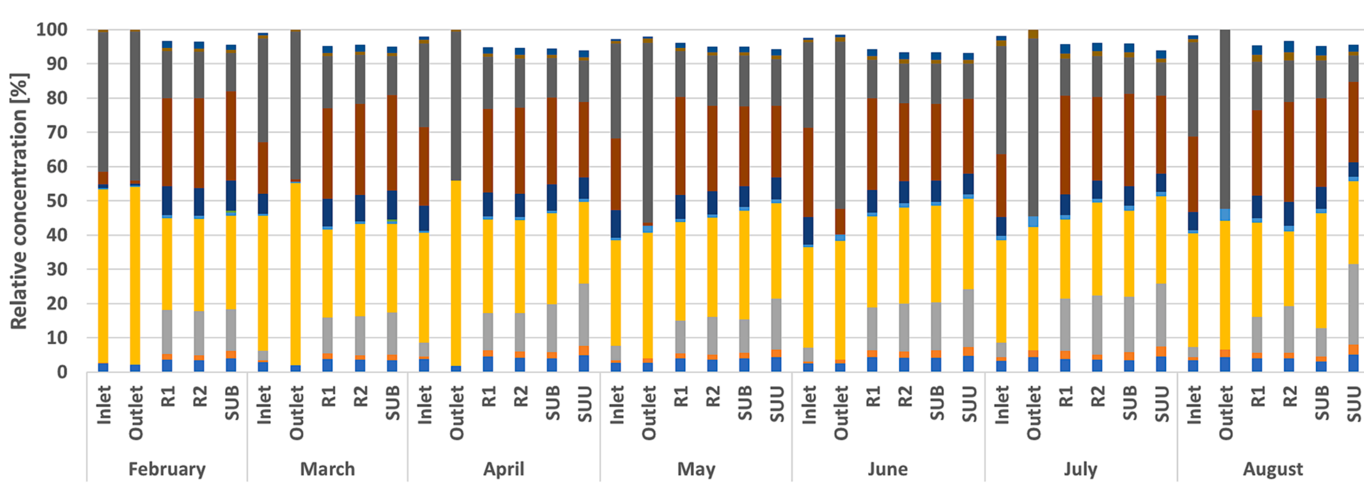


Fig. 5. Variation of the FAMEs composition in the inlet, outlet, and individual bioreactors (R1, R2, SUB and SUU) during the operational period.

organic material would be used for biodiesel production. According to Frkova et al. (2020), the potential loss of biogas production (anaerobic digestion), due to FOG extraction from sludge to produce biodiesel, is around 13–18 %.

Another challenge concerns the outlet wastewater from the LAT being fully treated by conventional processes. Microorganisms present in AST, as expected in a well working process, remove nutrients from wastewater while growing on them and producing the outlet water with the required quality to be discharged into water bodies. The average values of removal of COD and BOD from wastewater by OMOs in the LAT were 74.8 % and 86.8 %, respectively (Table 4), which would lead to a lower C/N ratio, which would presumably cause problems for nitrogen removal within the denitrification process in the AST (Sobieszuk & Szewczyk, 2006). In addition, the removal efficiencies of TN and TP were only 54.6 % and 5.8 %, respectively, which confirm that the LAT outlet wastewater must be fully treated by conventional WWTs to achieve the required quality for discharging into water bodies. Therefore, AST plants would need to deal with such depleted influent wastewater to meet the effluent requirements (Consul of the European Communities, 1991).

Consequently, substrate depletion must be considered in the LAT dimensioning to treat only a percentage of the inlet wastewater and be installed on a side stream. A techno-economic assessment of the production of biodiesel from sewage based on the LAT data assumed that only 10–15 % of the inlet wastewater would be used to produce biodiesel (WOW-Interreg, 2021).

3.4. Engineering recommendation for future design improvement

The design of the bioreactors and operational parameters worked very well and offered optimum conditions for growth of OMOs and lipid accumulation. Further tests at different WWTPs with higher COD loads would be desirable for up-scaling.

The main purposes of the separation unit were achieved. The sludge settleability was good overall during the entire operation period, the same applied to the biomass recirculation and sludge recovery for biodiesel production. It would be desirable to install a semi-continuous automated skimmer on top of the sedimentor for a consecutive harvest of the rich foam, and turbidity sensors installed in the different depths of sedimentor for better process synchronization. A sludge drying process and biodiesel quality assessment was not part of this work. However, some improvements may be envisaged, such as technologies allowing collection, dewatering and pre-drying of the sludge which should be more economically viable and practical.

As suggested in the techno-economic assessment of LAT, both technological and economic parameters may be further optimized (WOW-Interreg, 2021) considering all steps including drying, extraction, transesterification and biodiesel purification and estimated minimum selling price of the biodiesel from LAT to be €1.59/kg, which was higher compared to the literature. However, an increase in sludge recirculation by 20 % reduced the biodiesel cost to the market average. An additional reduction of capital expenditures by 50 % (e.g., fabrication of tanks from more economic material) reduced the production cost by 23 %. Further savings could be achieved with increasing the plant size along with lowering the operating expenses per unit plant capacity.

4. Conclusions

The pilot-scale LAT, in line with the literature, offered optimum growth conditions for *M. parvicella* and, consequently, the accumulation of lipids. The extracted lipids from bioreactors were three-fold higher than those already existing in sewage; biofuel and biodiesel production were 31 and 12 t/year, respectively. These values are expected to be even higher at other urban WWTPs, with higher loads of COD.

Deeper investigations into the growth and role in lipid accumulation of OMOs may be desired to increase the process effectivity. Full characterization of the biofuel composition could enlarge the pallet of renewable resources from sewage.

Table 4
Average concentrations of macropollutants in the inlet and outlet for the entire operational period (n = 29) ± standard deviation and the removal efficiency of LAT (%).

Macropollutants	Concentration ± Std. Dev. [mg/L]		Removal [%]
	Inlet	Outlet	
COD	**179.2 ± 72.4**	**72.4 ± 45.1**	**74.8**
BOD_5	**103.6 ± 48.8**	**48.8 ± 13.7**	**86.8**
NH^{4+}	17.1 ± 6.0	6.0 ± 3.1	81.8
NO^{3-}	0.5 ± 0.5	0.5 ± 4.7	−895.3
TN	**25.5 ± 9.0**	**9.0 ± 11.6**	**54.6**
Ortho-P	1.6 ± 0.3	0.3 ± 2.3	−44.7
TP	**3.0 ± 2.0**	**2.0 ± 2.8**	**5.8**

CRediT authorship contribution statement

Fernanda Cristina Muniz sacco: Writing – original draft, Writing – review & editing, Formal analysis, Methodology, Conceptualization, Investigation, Visualization, Data curation. **Zuzana Frkova:** Methodology, Conceptualization, Investigation, Writing – review & editing, Formal analysis. **Silvia Venditti:** Funding acquisition, Project administration, Writing – review & editing. **Carlo Pastore:** Writing – review & editing. **Cedric Guignard:** Writing – review & editing. **Joachim Hansen:** Supervision, Funding acquisition, Project administration, Writing – review & editing.

Declaration of Competing Interest

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

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.biortech.2022.128498.

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