Comparison of the mesophilic, thermophilic and temperature-phased anaerobic digestion of sewage sludge

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Abstract

Anaerobic digestion (AD) is currently spread all over the world for sewage sludge stabilisation, volume reduction and green energy production. Its implementation contributes to turn a wastewater treatment plant (WWTP) into a resource recovery facility.

Some of the main parameters of the AD process are the reactor temperature and configuration. There are three possible temperature ranges, namely psychrophilic (0-20 ºC), mesophilic (35-40 °C) and thermophilic (50-60 °C). The system's configuration can be of single-stage, double-stage or even triple-stage process. The single-stage AD consists of one reactor where all four steps of AD (hydrolysis, acidogenesis, acetogenesis and methanogenesis) go on at the same time. In a double-stage process, the hydrolytic and acidogenic steps take place in the first reactor, called fermenter, usually run under thermophilic conditions, while the acetogenic and methanogenic steps take place in the second reactor, normally run under mesophilic conditions. Hence, this double-stage process is called temperature-phased anaerobic digestion (TPAD) and consists of two stages which are carried out in two anaerobic reactors implemented in series.

AD processes at WWTP are studied quite well, however, there are still some issues that are open for further investigation. In this PhD Thesis, three different AD configurations were studied with regard to the following operational and functional issues:

- Digested sludge quality, dewaterability and pathogenic safety;
- AD efficiency in terms of mixing equipment and its operational regime;
- Life cycle assessment (LCA) of sludge and wastewater treatment.

Most often, anaerobic digesters are run in one stage (as it has lower capital cost) and under mesophilic conditions (as this temperature regime is supposed to be more stable). However, it is known that thermophilic conditions are advantageous over mesophilic ones in terms of methane production and digested sludge quality, mainly its pathogenic safety and dewaterability, which is the ability to „loose" water adsorbed by sludge flocs and trapped in among them. TPAD is a combination of above mentioned temperature regimes. It is a double-stage AD system that is supposed to combine the benefits of both by selecting the optimal hydraulic retention time (HRT) and organic loading rate (OLR) for each reactor.

Though TPAD has already been studied, a comprehensive study on the simultaneous operation of several AD systems fed with the same substrate and comparing not only the main operational AD parameters such as organic matter degradation and methane production rate, but also digested sludge quality in terms of dewaterability,

pathogenic safety and energetic value, considering its final disposal step, is still missing.

Thus, one of the objectives of this PhD Thesis was to compare the mesophilic anaerobic digestion (MAD), thermophilic anaerobic digestion (TAD) and TPAD digested sludges and define the best alternatives for final disposal. The complex parameter of dewaterability was tested by two methods, centrifugation and mechanical pressing. Applying both methods, the experimental results showed that TAD and TPAD overcome MAD performance. Indeed, TPAD seems to possess the most beneficial operational conditions. Firstly, TPAD has highest methane yield and organic matter destruction, hence the lowest energetic value. Secondly, TPAD digested sludge got the most favourable dewatering ability: the repeatability of dewaterability tests was as high as for MAD digested sludge, the polymer consumption was the same as for MAD digested sludge, while the sludge cake concentration was almost as high as for TAD digested sludge and so was the high pathogenic safety.

The AD efficiency depends on many factors, including the mixing system and its operational regime. A mixing system and its operational regime are of high practical interest, as the stirring process can affect the AD efficiency significantly, both positively and negatively. Hence, there are several studies that investigate the effectiveness of the AD process in terms of mixing efficiency. However, most of them focus on certain factors (like microbial diversity or organic matter degradation efficiency) under a single temperature regime and configuration without a parallel comparison of several AD systems. Therefore, it is not possible to compare different types of AD systems operated simultaneously and fed with the same substrate under different temperature regime and configuration. Thus, in this PhD Thesis two different types of mixing mechanisms (a simple flat one-straight-blade paddle agitator located at the bottom of the reactor and a more complicated two-straight-blade paddle impeller system) with two rotational regimes (95 rpm for the fermenter and 100 rpm for other three reactors and 30 rpm for the fermenter and 50 rpm for the others) were selected. These mixing equipment alternatives were tested simultaneously on three laboratory AD systems with three different temperature regimes (mesophilic, thermophilic and temperature-phased) and two configurations (single- and double-stage AD) and afterwards modelled. The experiments showed that the simplest mixing mechanism at slow mixing velocity affected the AD efficiency in a better way than the more complicated mixing system at higher rotational speed.

Another issue studied was a short HRT of the first stage of the TPAD system. It was found out that even at two days of HRT at TPAD1 methane content and volume can be reached in the fermenter and maintained a at significant amount at the second stage (TPAD2). These findings were proved by the microbiological analysis of samples taken from both stages of TPAD systems.

Finally, the same three alternative anaerobic digestion systems (TAD, MAD and TPAD) were compared to determine which system may have the best environmental performance. Two life cycle assessments were performed considering: the whole WWTP (for a functional unit (FU) of 1 m^3 of treated wastewater), and the sludge line (SL) alone (for FU of 1 m^3 of produced methane). The data for the LCA were obtained from previous laboratory experimental work in combination with full-scale WWTP and literature.

According to the results, the WWTP with TPAD outperformed those with TAD and MAD according to the majority of analyzed impact categories (i.e., Human toxicity, Ionizing radiation, Metal and Fossil depletion, Agricultural land occupation, Terrestrial acidification, Freshwater eutrophication, and Ozone depletion), except for Climate change where the WWTP with MAD has a 7% lower impact than with TPAD. In the case of the SL alone, the production of heat and electricity (here accounted for as avoided environmental impacts) led to credits in most of the analyzed impact categories except for Human toxicity. The best AD alternative was TAD concerning all environmental impact categories, except for Climate change and Human toxicity.

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List of abbreviations and symbols

AD	anaerobic digestion
C_2, C_3, C_4	acetic acid, propionic acid, butyric acid
CFD	computational fluid dynamic
CST	capillary suction time
D1	biomass sample taken from the central part of the reactor TPAD1 (suspended biomass)
D2	biomass sample taken from the reactor wall in TPAD1 (attached as a biofilm)
D3	biomass sample taken from the central part of the reactor TPAD2 (suspended biomass)
DNA	deoxyribonucleic acid
DS	dissolved solids
EDC	endocrine disrupting compound
EOD	electrodewatering
EPS	extracellular polymeric substance
GT	gravity table
GC	gas chromatography
FA	free ammonia
FISH	fluorescence in situ hybridization
FU	functional unit
HRT	hydraulic retention time
LCA	life cycle assessment
M	mesophilic
MAD	mesophilic anaerobic digestion
NSAID	non-steroidal anti-inflammatory drug
OHPA	obligate hydrogen producing acetogens
OLR	organic loading rate
ORP	oxidation reduction potential
PE	people equivalent
PHA	polyhydroxyalkanoate
(q)PCR	(quantative) polymerase chain reaction
RNA	ribonucleic acid
sCOD	soluble chemical oxygen demand
SL	sludge line
T	thermophilic
TAD	thermophilic anaerobic digestion
COD	total chemical oxygen demand
TAN	total ammonia nitrogen
TPAD	temperature phased anaerobic digestion
TPAD1	the first stage of TPAD
TPAD2	the second stage of TPAD
TS	total solids
TSS	total suspended solids
USEPA	US EPA: United Sates Environmental Protection Agency
VFA	volatile fatty acid
VDS	volatile dissolved solids

VS	volatile solids
VSS	volatile suspended solids
VSS_{rem}	volatile suspended solids removed
WAS	waste activated sludge
WRRF	wastewater resource recovery facility
WW	wastewater
WWTP	wastewater treatment plant

Chapter 1 – Introduction

1.1 Problem statement

Nowadays, up to 46% of the world population has a lack of safe sanitation services, and the Sustainable Development Goal 6 (Clean Water and Sanitation) aims to ensure access to water and sanitation for all by 2030. Reaching this target is only possible considering new wastewater treatment plants (WWTP) construction and rehabilitation of existing ones, including solutions for sustainable sewage sludge management.

Currently, sustainable sewage sludge management tends to the implementation of a resource recovery approach. It turns WWTPs into water resource recovery facilities (WRRF) (Zhao *et al.*, 2019; Lanko *et al.*, 2020). Hence, the sludge is converted into energy, nutrients, and other valuable substances (metals, specific organic substances). All of the above mentioned can be reused in different spheres of our life including agriculture (fertilizers), various industries (biopolymers, fuels), communal services (heat) (Lanko *et al.*, 2020; Kominko *et al.*, 2019; Pauline *et al.*, 2021). By this, a better environmental protection level is achieved. (Seleiman *et al.*, 2021).

Sewage sludge from activated sludge WWTP comprises the so-called primary sludge, produced during the primary wastewater treatment in sedimentation tanks; and secondary sludge, produced during the secondary wastewater treatment in biological reactors as a result of microbial growth. Normally, the ratio of the produced sludge types may vary from 40 to 70% of primary to secondary sludge (Ferrer *et al.*, 2010; Markis *et al.*, 2016). At relatively small WWTPs (<50,000 PE) the primary sedimentation step may be absent, having only secondary sludge production. Secondary sludge is also known as waste activated sludge (WAS) (Liu *et al.*, 2021).

The recovery of resources and final reuse may cover around 30% of the costs for sewage sludge handling (Chen *et al.*, 2021), which is an important amount given that the sewage sludge handling usually takes up to 50% of the wastewater treatment expenses (Neczaj *et al.*, 2019).

One of the most common way to treat the sewage sludge regularly produced at conventional (based on activated sludge system) WWTP is anaerobic digestion (AD).

AD consists of the biodegradation of organic matter under anaerobic conditions, leading to the production of biogas (mostly composed by methane) and a stabilised digestate (Jiang *et al.*, 2022).

Any AD possesses certain operational parameters that define the expected outcoming results (a balance of energy efficiency, digested sludge quality, namely, dewaterability, health safety, etc.) and possible final disposal of digested sludge (landfilling, incineration, agricultural application).

Among these operational parameters the most important are temperature, substrate composition, hydraulic retention time (HRT), organic loading rate (OLR), feeding pattern and mixing regimes. In addition, since recently, anaerobic digester configuration (single- and double- stages) is one of the basic and, at the same time, crucial parameters that is laying in a background of AD process itself, which was also considered in this study. Between two single-stage AD systems, mesophilic anaerobic digestion (MAD) and thermophilic anaerobic digestion (TAD), there are known concepts that in terms of methane production and organic matter degradation TAD is superior over MAD, however, MAD is more stable in operation and its digested sludge demands less flocculant for dewatering. Simultaneously, the double-stage AD, so-called, temperature-phased AD (TPAD) configuration complies the advantages of both single-stage AD systems. Hence, there are different recent studies revealing benefits and bottlenecks of each of them in terms of AD efficiency, but the data on sludge digested sludge quality is insufficient. There are left unclear data on dewaterability, pathogenic safety, energetic value of digested sludge after single-stage systems (MAD and TAD) and TPAD. In addition, there are just a few studies performed regarding comprehensive approach and complex investigation of TAD, MAD and TPAD simultaneous operation and their influence on environment using specific analytical tool, life cycle assessment.

1.2 Objectives

This study aimed to compare three types of AD systems (MAD, TAD, TPAD) in terms of methane production, digestate quality and potential environmental impact.

The specific objectives were as follow:

– to evaluate the effect of different mixing systems on the production of biogas and methane;
– to assess the influence of AD configuration and selected HRT on digested sludge quality (dewaterability, calorific value and pathogenic safety);
– to compare the potential environmental impact of the studied AD processes using the Life Cycle Assessment (LCA) methodology.

1.3 Scope and organization of the thesis

The thesis begins with the **theoretical background** of the research topic, which leads to the **research goals**.

Chapter 2 is devoted to the **Materials and Methods**. It describes the laboratory installation, all experimental phases – **Experimental set-up**, analytical methods – **Analytical tests: Description and Significance**. In addition, for the results validation was used **computational fluid dynamics** (CFD) **modelling**, **statistics** and **life cycle assessment** (LCA).

Chapter 3 is devoted to the results, which respond to the three research goals, and are presented in three subchapters:

1) The first subchapter concerning **the mixing efficiency in AD**;
2) The second subchapter sheds the light on **a digested sludge quality (mainly, dewaterability) and its balance with the methane production** as one of the key parameters of AD efficiency;
3) The third subchapter shows the results of the **LCA** for the studied AD processes.

The overall conclusions and further research perspectives are summarized in the Chapter 4. General conclusions.

1.4 Theoretical background

1.4.1 Description of AD process

The fact that the decaying organic matter generates inflammable gases forms the principle of anaerobic digestion. First discovered in the 17th century by Jan Baptita Van Helmont (Abassi *et al.*, 2012), the concept rapidly and controversially gained popularity. In 1881, French scientist Louis H. Mouras was the first to apply anaerobic digestion to wastewater treatment using a simple septic tank which he named "automatic scavenger" (Abbasi *et al.*, 2012). In 1905, the Imhoff tank designed by Karl Imhoff was the first anaerobic reactor in which a single tank was used to stabilize solid sediments. However, it was in 1927 in Germany that the Ruhrverband, Essen-Relinghausen developed the concept of controlled digestion of entrapped solids by installing a sludge-heating apparatus in a separate distinct reactor (Henze *et al.*, 2008). Over the passing century, developing research on optimization of anaerobic digestion made it one of the most common techniques to treat sewage sludge across the globe. This success is attributed to the phenomenal extent of environmental and economic solutions it introduces to wastewater treatment industry (Figure 1.1) with the clean fuel that it generates, biogas.

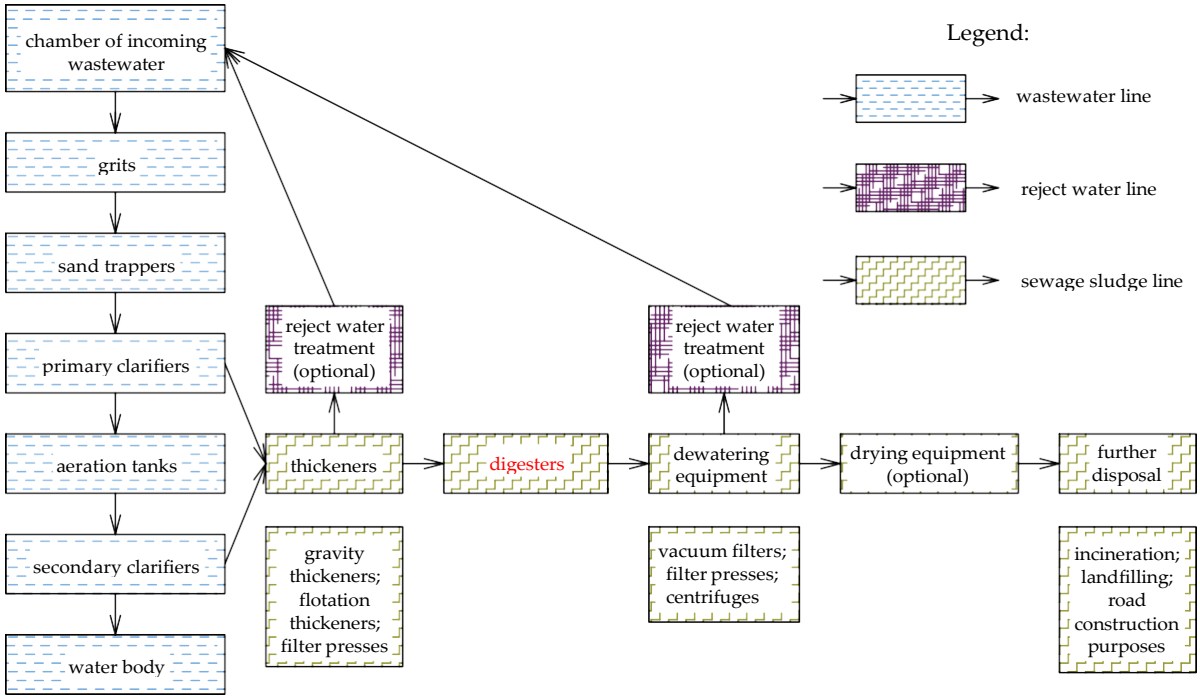


Figure 1.1 – The classic scheme of wastewater treatment plant

The Figure 1.1 shows a scheme of conventional WWTP with defined two main lines: 1) wastewater treatment (WW) line that consists of mechanical and biological steps untill it discharges to the water body; 2) sewage sludge (SL) line that is presenting all steps needed for complete sewage sludge handling. The additional line of reject water is depicted to highlight its significant influence on WW line and close interconnection of two main lines of WWTP.

Henze *et al.* (2008) define anaerobic process as the fermentation process in which biogas is produced from degradation of organic matter. It successfully removes biodegradable organic compounds and leaves behind mineralized compounds such as PO_4^{3-}, S^{2-}, NH_4^+ in the solution. Anaerobic digestion primarily follows a sequence of four major microbiological process: hydrolysis, acidogenesis, acetogenesis and methanogenesis (Figure 1.2).

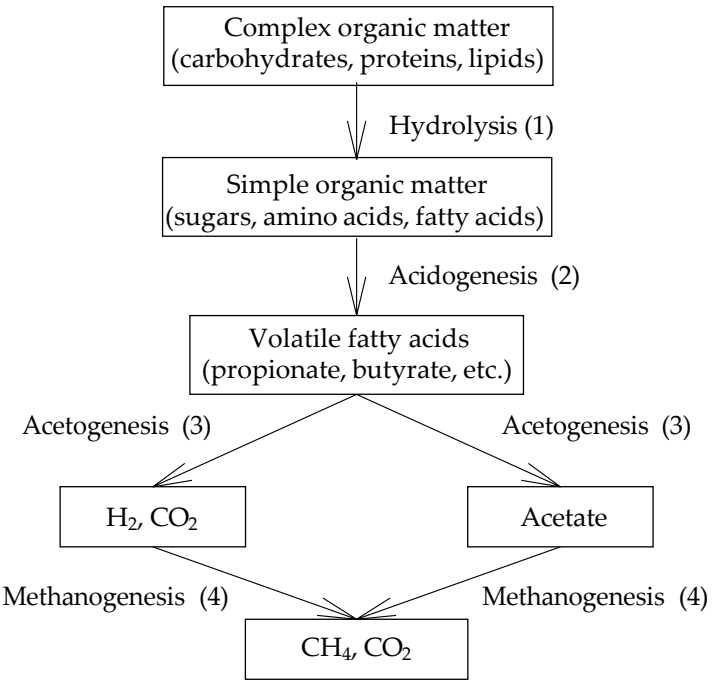


Figure 1.2 – Schematic representation of anaerobic digestion: Processes, products and participating microorganisms

It occurs under strictly anaerobic conditions defined by an oxidation reduction potential (ORP) below -200 mV (Appels *et al.*, 2008) and is strongly dependent upon the extent of activity carried out by a complex combination of microorganisms, mainly archaea and bacteria to convert organic matter into methane (CH_4) and carbon dioxide (CO_2) predominantly.

During hydrolysis, insoluble organic material and higher molecular weight compounds or polymers like polysaccharides, proteins, lipids, fats etc. undergo enzyme-mediated degradation into simple organic monomers and oligomers such as amino acids, monosaccharides and fatty acids. Strict anaerobes such as *clostridia*, bacterides and facultative bacteria like *streptococci* participate in this step. Although anaerobic digestion is a sequential process, hydrolysis is usually considered the rate limiting step. Henze *et al.* (2008) argue that this is not because of scarce enzymatic activity but rather availability of freely accessible surface area of particles and overall structure of the solid substrate.

Monomers formed in hydrolytic phase are then transformed into short and medium chain organic acids, alcohols, hydrogen, CO_2, ammonia (NH_3) and other byproducts by acidogenic bacteria in the acidogenesis phase. Unbalanced acidogenesis can lead to acidification of digesters causing process failure. These higher acids and alcohols are further reduced to methanogenic substrates, mostly acetate, CO_2 and H_2 by acetogens in the next step of acetogenesis. Two types of acetogens, namely obligate hydrogen producing acetogens (OHPA) and homoacetogens are involved in this process. Acetate formation acts as an immediate precursor for majority of the methane

formation. In the final metabolic step of methanogenesis, methanogens (all archaea) produce methane from acetate, H_2 and CO_2. Hydrogentrophic archaea utilize hydrogen as elector donor and CO_2 as electron acceptor to form methane, whereas acetoclastic archaea split acetate into methane and CO_2.

During acetogenesis, usage of protons as electron acceptors leads to formation of H_2. At high partial pressure of H_2, inhibition of acetate production occurs because oxidation of acids to acetate can only occur at low partial pressure of H_2. Hydrogentrophic methanogens continuously consume H_2 to produce methane, and, therefore, an interspecies hydrogen transfer maintains this symbiotic relationship in anaerobic digestion.

1.4.2 Mesophilic and thermophilic single-stage digesters

Anaerobic digestion can be carried out at two different ranges of temperatures namely mesophilic which is between 35 ^{0}C and 40 ^{0}C, and thermophilic that is between 50 ^{0}C to 57 ^{0}C (Qasim *et al.*, 1999; Lin *et al.*, 2018) and more appropriate for thermophilic archaea. Temperature plays a key role in anaerobic digestion and can greatly influence the kinetics, effluent quality, stability, conversion and the methane yield of the process (Sanchez *et al.*, 2001).

Most WWTPs operate digesters at mesophilic temperatures (Qasim, 1999) primarily due to lower energy demand. Mesophilic anaerobic digestion (MAD) is also preferred in terms of process stability because thermophilic archaea are more sensitive and respond much more significantly to any alterations in operational conditions such as change temperature, organic loading rate or characteristics of the influent sludge (Kim *et al.*, 2002; van Lier, 1996). Thermophilic anaerobic digestion (TAD) appears advantageous in terms of higher performance in reduction of volatile solids, better destruction of pathogens and higher growth rate of microorganisms, increased biochemical reactions and positively stimulated hydrogen transfer which results in an escalated methanogenic potential at lower hydraulic retention times (Zabranska *et al.*, 2000).

Additionally, TAD provides a heightened hygienization effect (Gavala *et al.*, 2003) and sludge produced from this process satisfies the criteria for Class A bio-solids according to the USEPA thereby considered suitable for land application (Watanabe *et al.*, 1997). However, the benefits of TAD over MAD are heavily dependent on the type of sludge to be digested and its conversion efficiency (De Vrieze *et al.*, 2016). De Vrieze *et al.* (2016) demonstrated that thermophilic digestion of sewage sludge also contributes to higher energy and nutrient recovery, although its economic feasibility rests on the size of the treatment plant and fate of future prices for energy and nutrients.

Although most literature presents TAD as more efficient in terms of maximum methane production, , destruction of pathogens and viruses harmful to public health (Kim *et al.*, 2002; Gao *et al.*, 2013), studies also show the many disadvantages it holds in comparison to MAD. It requires higher operational energy, produces low quality supernatant with large dissolved solids, exhibits poor formation of floc and dispersed particles, poor effluent quality, turbidity (Appels *et al.*, 2008; Suvilampi *et al.*, 2005) and process stability due to high volaile fatty acids' concentrations (Kugelman *et al.*, 1989). Kim *et al.* (2002) observed that in the start-up period of their comparative study, both temperatures showed efficient and stable performance acid producers (acidogens) and consumers (acetogens). Based on this, the authors highlight the importance of close microbial community to upgrade the microbial consumption of dissolved hydrogen. Thus, they suggest that disadvantages of TAD can be countered by the utilization of close microbial consortia proximity. A study by Song *et al.* (2004) reported better effluent quality in MAD and attributed this to the low substrate affinity of thermophilic organisms.

1.4.3 Temperature-phased anaerobic digestion

Whereas AD can be implemented with the variations in temperature, it can be also introduced in different configurations (sinle-, double-, triple- stage reactors) (Leite *et al.*, 2016; Micolucci *et al.*, 2018; Liu *et al.*, 2019). Normally, all four stages of the AD process, namely hydrolysis, acidogenesis, acetogenesis and methanogenesis (Cao *et al.*, 2020), take place in the same reactor in the case of single-stage AD. Separate functioning of the first thermophilic (hydrolytic and acidogenic) and second mesophilic (acetogenic and methanogenic) stages in double-stage AD is accomplished to overcome the drawbacks of single-stage systems (Cao *et al.*, 2020; Srisowmeya *et al.*, 2020). Recent developments in anaerobic digestion showed the introduction of temperature-phased digestion (TPAD) wherein a sludge exchange is established between spatially separated thermophilic and mesophilic digesters (Figure 1.3).

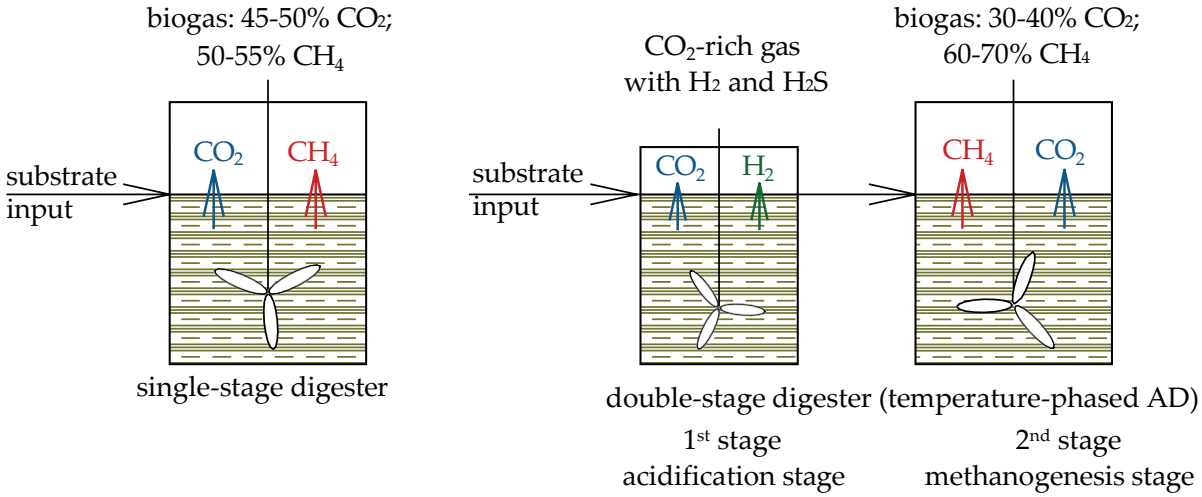


Figure 1.3 – Two types of anaerobic digestion systems: single-stage (thermophilic or mesophilic) and double-stage (temperature-phased anaerobic digestion)

As mentioned, the most widely used anaerobic digester is a mesophilic single-stage, since its operation is known as the most simple and stable (Kim *et al.*, 2002; Zhang *et al.*, 2018). Nevertheless, the tendency changes towards more metabolically efficient and pathogenically safe digesters such as thermophilic single-stage or temperature-phased double-stage reactors (Song *et al.*, 2004).

Song *et al.* (2004) reported in their study that similar to thermophilic digestion, co-phase system attained up to 98.5-99.6% total coliform and pathogen destruction. However, the decrease in volatile solids was much more than that of a single-stage thermophilic digester. Song insists that individual benefits of single-stage mesophilic and thermophilic digestion can be achieved together in a temperature-phased system. The study observed higher specific methane yield, process stability and effluent quality in regards with soluble COD and VFA concentration in the TPAD system. This result is explained by sharing of nutrients and intermediates by anaerobic microorganisms, their selection of active and higher substrate affinity and efficient functioning of anaerobic digestion as a consequence of sludge exchange between mesophilic and thermophilic digesters. Samaras *et al.* (2014) also observed a higher destruction of endocrine disrupting compounds (EDCs) in the temperature-phased AD where most micropollutants were removed in the thermophilic digester. Although no significant improvement was observed in the removal of non-steroidal anti-inflammatory drugs (NSAIDs).

Another study by Yu *et al.* (2014) showed that in TPAD1 production efficiency maximum was attained at an OLR of 5 kgVS/m^3.d and above this OLR the process efficiency reduced for both digesters. However, biogas production rate remained high and the methanogenic process did not collaps even at an OLR of 10 kgVS/m^3.d.

Hence, TPAD represents a combination of thermophilic and mesophilic single-stage digesters which performs with better stability than thermophilic and higher organic matter degradation rate than mesophilic, which means increased energy efficiency and better control of process parameters (Fernández-Rodríguez *et al.*, 2016; Srisowmeya *et al.*, 2020).

1.4.4 Mixing process in anaerobic digestion

1.4.4.1 Mixing as a factor of AD efficiency

There are several recent studies that focus on the stirring effect on the AD process (Kariyama *et al.*, 2018; McLeod *et al.*, 2019). Many researchers evaluated the influence of parameters like suspension formation, homogenisation, temperature equalisation, microbial floc formation as they are main functions of the process with regard to the mixing effect (Lindmark *et al.*, 2014; Nguyen *et al.*, 2019). In addition, there were

certain studies that assessed the stirring effect on double- and even triple-stage AD systems (Ma *et al.*, 2019; Liu *et al.*, 2019). However, in most studies, different mixing aspects (such as the amount of paddles, their location, reactor design, stirring regime) were either omitted, or slighty investigated. That led to the problems with defining and confirming if the mixing process was in correlation or causation with major operational problems, for instance, as the reason of intensive foaming or not sufficient organic matter degradation and methane production (Lindmark *et al.*, 2014; Subramanian & Pagilla *et al.*, 2014).

It is important to mention that energy consumption for mixing purposes is a big part of expenses for AD, which makes out of it a subject for further investigation (Meister *et al.*, 2018).

1.4.4.2 Stirring mechanisms and their working parameters

There are several well-known ways and correspondent mechanisms of mixing in the AD reactors: biogas recirculation, impeller mixing, and sludge recirculation (Karim *et al.*, 2005). Among them, the impeller mixing is the most energy efficient and common one (Wu *et al.*, 2010; Lindmark *et al.*, 2018).

There are a lot of scientific discussions (Stroot *et al.*, 2001; Karim *et al.*, 2005; Wu, 2014; Kress *et al.*, 2018) regarding the optimal mixing conditions in terms of the most important aspects of digester mixing which are the intensity, interval and duration of mixing. According to the above mentioned research works, the intensity of stirring is less influential than its interval and duration. However, there is still no proper road mapping towards optimal mixing conditions for different substrate content, its TSS and OLR (Zhai *et al.*, 2018).

The different types of mechanical stirrers are also studied (Lemmer *et al.*, 2013; Kariyama *et al.*, 2018), as the impeller type and geometry are important in saving energy and also in improving the mixing quality and avoid sludge partition, temperature stratification, crust formation and other negative consequences in the context of AD efficiency.

Therefore, this thesis compared a simple flat one-straight-blade paddle agitator located at the bottom of the reactor with a more complicated two-straight-blade paddle impeller system, in three AD systems, namely, MAD, TAD and TPAD treating the same substrate (WAS). The tested parameters were the type of mechanical stirrer and the stirring intensity (speed, rpm).

1.4.5 Digested sludge quality and its assessment

1.4.5.1 Digested sludge quality parameters

In conventional activated sludge WWTPs, excess sludge is continuously formed at the biological reactor of the wastewater treatment line. WWTP operational expenses to handle the produced excess biological sludge, namely waste activated sludge (WAS), together with primary sludge may go up to 50% (Leite *et al.*, 2016; Wei *et al.*, 2018; Yang *et al.*, 2019). It has been a long time since AD was adopted as one of the most effective solutions of sewage sludge treatment in terms of sludge reduction, stabilization and resource recovery (Lamnatou *et al.*, 2019; Rajendran *et al.*, 2019; Wainaina *et al.*, 2020). The sludge reduction is highly influenced by the quality of the sewage sludge, namely its rheological properties (viscosity, shear rate, floc strength), surface charge, water content, hydrophilicity/hydrophobicity, foaming potential (Liang *et al.*, 2020). All together these properties describe the digested sludge and effort that has to be made to remove the water from the digested sludge as much as it is economically efficient. Based on the resulting values of digested sludged and dewatered sewage sludge, its final disposal can be decided on. Depending on some other quality parameters of the sludge, such as total solids content, heavy metals' and toxic substances' content and the pathogenic safety, the digested sludge can be reused as a biofertilizer, biofuel or construction material for roads (Chen *et al.*, 2021), rather than disposed of in landfill sites.

1.4.5.2 Dewatering process

Normally, the performance of AD systems is assessed by organics degradation efficiency and methane production. However, "to close the loop" of digestion efficiency, digested sludge quality data assessment is needed. Such digested sludge properties as its dewaterability is a complex parameter, therefore, there is always a shortage of its evaluation data.

Dewaterability being a complex quality parameter of sewage sludge describes the ability of sludge flocs to "loose" water which is entrapped in there. According to the difficulty of its removal all water that is present in the sewage sludge can be segregated into free water and bound water. The free water which is unaffected by solid particles. In its turn, bound water can be divided into two types: interstitial water that is physically trapped inside space between particles and surface water which is adsorbed onto the surface of solid particles (Wei et al., 2018).

Free water can be removed without much effort through thickening or mechanical procedures using gravity and pressure forces. However, bound water exists in a floc matrix and can be removed only by thermal drying at above 105^0C (Jin, *et al.*, 2004). Neyens & Baeyens (2003) consider bound water as one of the main limiting factors in water removal efficiency, mentioning that some bound water cannot be removed at all. Qi *et al.* (2011) state that the challenges faced in dewatering are majorly due to presence of colloidal materials and high organic content in sludge solids.

Dewatering of biosolids is indispensable in order to reduce the water content of the sludge and consequently its overall volume to facilitate transport, handling and final disposal or to minimize the energy and space required in case of incineration and drying. There are a lot of different technologies of sewage sludge dewaterability assessment. Among them are mechanical (centrifuging, belt-, screw- and filter-pressing), thermal (convective, conductive, radiative drying), electrical (electro- and electrochemical) dewatering. The mechanical methods are well spread, meanwhile the thermal methods are used as additional ones after the mechanical in order to imrove the sludge quality or as the pre-treatment technology before its incineration (Ma *et al.*, 2021). Electrodewatering is a modern technique which uses an electric field to run a pressure-driven dewatering operation resulting in quicker dewatering kinetics and increased total solids' contents in the final cake through electroosmotic water transport (Saveyn *et al.*, 2006).

1.4.5.3 Dewaterability: Influencing factors

The factors associated with dewaterability include the composition and particle size of the substrate (Shao *et al.*, 2009); sludge properties (Yang *et al.*, 2019); operational temperature and floc characteristics (Suhartini *et al.*, 2014). During anaerobic digestion, flocs disintegrate first and extracellular polymeric substances (EPS) are released into the bulk solution during hydrolysis and acidogenesis. Many studies account EPSs and their quantity in the sludge as one the biggest influencing factors for sludge dewaterability (Shao *et al.*, 2009; Yu *et al.*, 2010; Zhou *et al.*, 2002). Shao *et al.*, (2009) reported that increase of organic matter in the slime fraction of the sludge could result in reduced dewaterability, whereas an increase in the pellet fraction could improve it. Thus, the spatial distribution and components of EPS significantly impact sludge dewaterability (Zhang *et al.*, 2015), and an excessive release of EPS decreases sludge dewaterability (Liang *et al.*, 2020).

Additionally, Li *et al.* (2019), Toutian *et al.* (2021) observed that dewaterability was highly decreased under alkaline conditions and faintly increased in acidic environment. An *et al.* (2017) suggest that the total solids contents can also influence dewaterability of the digested sludge alongside organic loading rate (OLR) and biogas yield. Numerous studies point out the importance of filter cake properties in dewatering. Thapa *et al.* (2009) suggest the measurement of sludge cake yield stress in order to quantitively analyze cake porosity, permeability and resistance to compression, all of which are major influencing factors. Citeau *et al.* (2011) studied the influence of salt and pH on electro-dewatering and found that the system performed better at slightly acidic pH and low salt concentration.

Zhou *et al.* (2002) consider digestion temperature as one of the leading factors that affect dewaterability. When Suhartini *et al.* (2014) compared the dewaterability of sugar beet pulp digested at thermophilic and mesophilic temperatures, they observed

a reduction in the filterability of the mesophilic digested sludge with a hydraulic retention time (HRT) of 55 days; whereas no decrease in dewaterability was seen in the thermophilic digested sludge. Other authors also demonstrated that well digested thermophilic sludge had a better ability to be dewatered, though it demanded higher flocculant consumption (Lloret et al., 2013). In another study, by Wang *et al.* (2017), MAD showed better dewaterability than TAD. In accordance with this, Wang *et al.* (2016) also achieved poor dewaterability and higher water retention for thermophilic sludge at high total solids content. These outcomes can be compared with similar results found by Chi *et al.* (2010) who also suggest the utilization of MAD in terms of enhanced dewaterability of digested WAS. In their experiment, they noted higher concentrations of soluble organic matter in the dewatered supernatant from TAD which was responsible for the poor dewaterability of thermophilic sludge and poor effluent quality from the process. However, the authors suggest that if the main aim is to reduce the final volume of sludge, TAD should be considered. Another example of temperaturę influence on dewaterability is thermal hydrolysis application, which improved the dewaterability (Barber *et al.*, 2016).

Hence, it can be said that the aiming higher methane yield and degradation rate, TAD looks better, however, MAD can outperform TAD when assessing the dewaterability and flocculant consumption for dewatering purposes. It should be noted that the quality of the sludge to "loose" the water is highly dependent on the substrate.

1.4.5.5 Dewaterability measurement

Efficient dewatering is challenging to accomplish and requires a deep understanding of dewaterability characteristics of the sludge, which may vary from one kind to another and consequently affect the extent of water removal from the sludge. Novak (2006) comments that the capacity of water removal is a reflection of the sludge dewatering property. Novak also recommends the gravity table (GT) value as a resourceful parameter to characterize the shear in the mechanical dewatering equipment. The GT value is the product of shear time and the mean of velocity gradient.

Kemira Kemi AB procedure is a two-step method where sludge is first filtered and then pressed to study its characteristics and was presented by Bouskova and Jansen (2006). They characterize dewaterability in terms of separability, which they describe as the extent to which particles are held together in the floc matrix; and compressibility which is described as the scope of the sludge to be dewatered under a certain pressure. Watson (1999) also agrees that due to the mechanical compression of sludge cake, compressibility is a significant characteristic that influences the amount of water obtained during dewatering and, hence, the dewaterability itself.

Dewaterability is measured using various other methods. Capillary Suction Time (CST) has been popularly adopted to asses dewaterability (Jin *et al.*, 2004). It measures the amount of time (in seconds) to travel a fixed distance on a particular filter paper. Poor filterability and dewaterability are reflected through high CST values. CST is simple, fast and cost effective but limits the possibilities to model and ergo predict physical properties such as the bound water content in the sludge (Chen *et al.*, 1996; Scholz, 2005).

Specific Resistance to Filtration is another method to express dewaterability in terms of compactibility of sludge flocs, although thought to be quite complicated (Tastu, 2007). Frozen Image Centrifugation can also be used to measure dewaterability, using the effect of freezing and thawing the sludge sample and the stroboscopic technique in which a 'frozen image' of the sample is generated. This allows changes in the solid liquid interface to be observed and measured in real time without stopping the centrifuge (Suhartini *et al.*, 2014).

Hence, there are many methods of sludge dewatering assessment. However, some of them have certain theoretical limitations (such as absence of possibility to predict the physical properties and processes in reality, for instance). Hence, in this thesis, were applied two methods similar to the ones used at full-scale WWTP.

1.4.6 Life Cycle Assessment

The zero-waste approach and circular economy paradigm change the angle at which nutrients, metals, organic matter and other substances from WAS can be converted into valuable materials like biofuels and biofertilizers.

Resource recovery processes are being widely adopted. For instance, at the moment, the attention is drawn to the reject water obtained after the AD process where the concentration of nutrients is significantly higher than in the influent wastewater (Khan & Nordberg, 2018). At the same time, the temperature regime and configuration of AD may significantly influence the nutrient concentration in the reject water and its volume (Leite *et al.*, 2016; De Vrieze *et al.*, 2016; Ruffino *et al.*, 2020). Thus, adopting different AD type the environmental picture of WWTP might be also different.

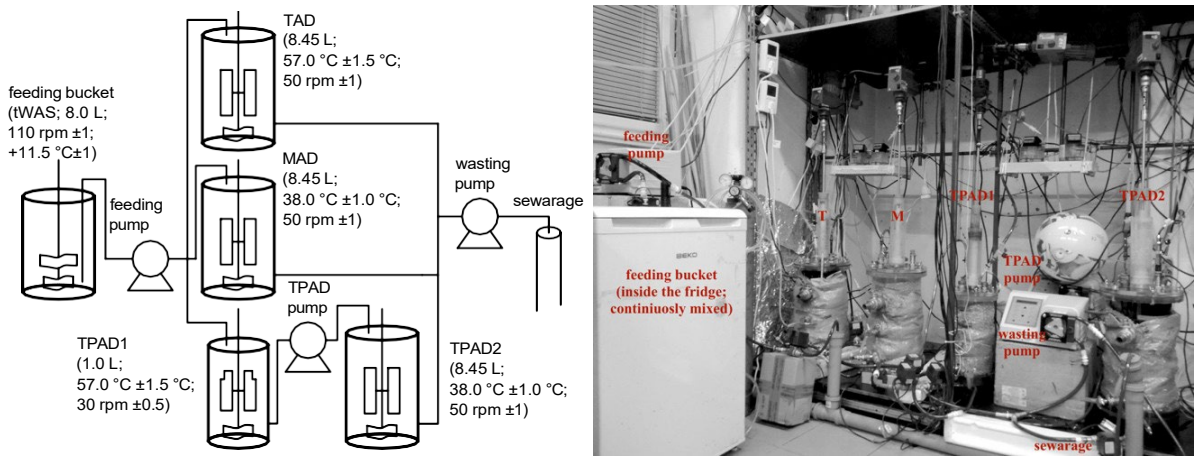
Despite the current exploitation of MAD and increasing interest in TAD and TPAD systems' application, there are few studies comparing the environmental impact of full-scale WWTP with different AD systems (Yu *et al.*, 2020) and none concerning the comparison of the environmental impact of all three of them. The environmental impact assessment would allow for defining which AD system is the most beneficial in terms of environmental protection.

LCA is an analytical tool, which allows to assess the environmental impacts of a product, technology or process according to the "cradle-to-grave" approach. This time-tested technique allows not only to evaluate the potential environmental risks, but also define the life cycle stage and type of environmental impacts. The application of this method may help to improve the studied product, technology or process by making its life cycle more friendly to the environment. LCA consists of four main steps according to ISO 14040 (2006), and ISO 14042 (2006): i) goal and scope definition; ii) inventory analysis; iii) impact assessment; and iv) result interpretation.

The LCA approach was used not only as a standard practice to estimate the environmental burden of the technological process (Li *et al.*, 2017; Zhang *et al.*, 2018; Li & Feng, 2018) on a micro level to compare the three AD options, but also as an alternative to build up a regulatory planning system on a meso level to increase the efficiency of project-level decision-making and to provide the advice on potential improvements for the sector's management, and also to ensure the realization of strategic environmental goals (Zhang *et al.*, 2020).

Chapter 2 – Materials and methods
2.1 Experimental set-up and procedures
2.1.1 Laboratory set-up

There were constructed three AD systems: single-stage thermophilic, single-stage mesophilic and double-stage temperature-phased system. The scheme and picture of the whole installation are presented in the Figure 2.4.



(a) (b)

Figure 2.4 – A principal scheme (a) and photo (b) of the laboratory installation

At each experimental period, the quality of the substrate was almost the same, as reported in the Appendix, Table A.1.3. The ambient temperature in the lab was the same during all experiments (24.0±1.5) °C.

2.1.2 Inocula sampling

The initial anaerobic sludge for laboratory reactor inoculation was sampled at the full-scale anaerobic digesters at the full-scale Czech wastewater treatment plants (WWTPs). The samples were evaluated in terms of: Total (Suspended) Solids (TS and TSS), Volatile (Suspended) Solids (VS and VSS), total and soluble Chemical Oxygen Demand (tCOD and sCOD, respectively). The analyses were conducted in technical triplicates according to the Standard Methods (APHA, 2012).

2.1.3 Substrate storage and characteristics

The thickened aerobic waste activated sludge (tWAS) was used as substrate for all AD systems during the whole period of experiments.

The substrate was sampled once per three weeks and stored in the thermostat at (8±0.5) ºC in the plastic containers. Once per two days certain amount of tWAS was put to the substrate bucket which was kept in another smaller fridge at (11.5±1.0) °C,

continuously mixed at 112±1 rpm. The shaft of the impeller, installed in the substrate bucket, had two paddles mounted on 4.0 cm distance from each other in order to provide the homogeneity to the whole volume of the substrate bucket (≒ 8.0 L) and prevent the substrate from drying out and forming the crust. The mixing process was performed by IKA RW 20 digital overhead stirrer with setting accuracy speed of ±1 rpm (60-2000 rpm; for quantity of up to 20 L of water).

A full depiction of substrate characteristics is presented in the Table 2.1.

Table 2.1 – The substrate characteristics over the whole experimental period

Substrate	TS (g/L)	DS (g/L)	TSS (g/L)	VS (g/L)	VDS (g/L)	VSS (g/L)	VSS/TSS (-)
tWAS	68.5 ±1.05	9.8 ±1.15	58.6 ±2.18	48.7 ±0.72	7.3 ±0.80	41.4 ±1.46	0.70±0.01

2.1.4 Reactor heating and temperature maintenance

The mesophilic reactor and mesophilic stage (the second stage, TPAD2) of TPAD stage had been running under temperature of 38±1.5°C, the thermophilic reactor and thermophilic stage (the first stage, fermenter, TPAD1) had been heated up to 57±1.5°C. The reactors were heated by tightly enclosing the outer reactor walls with the heating elements. The temperature regimes were maintained by Sygonix tx. 3 indoor thermostat adapter working in 24-h mode in the range from -20°C to 70°C with the hysteresis of ±0.2°C to the set value. The pH and temperature online data were downloaded and logged from the pH-probes Polilyte Plus H VP 225 PT1000.

Thus, there were two temperature sensors allowing to monitor the temperature in every reactor (Appendix A, Figures A.1.1 and A.1.2). The first sensor was used as an indicator for the thermostat, and it was installed closely to the reactor bottom (around 6.0 cm – for TPAD1 and 11.0 cm – for the other reactors). The second probe measuring the medium temperature was a pH-probe with incorporated thermometer. This sensor was placed on the depth of 16.5 cm from the inner surface of every reactor lid.

2.1.5 Automatic feeding

The feeding and wasting processes were automated, and governed by LabView 2012 software ("National Instruments", the Czech Republic). There were three programmed cased drive tube pump Verder Flex Vantage 3000 P R3I EU, "The Verder Group", the Netherlands: the first one – the feeding pump; the second one – TPAD pump as it was transfering pre-digested sludge from the first stage of TPAD to its second stage; the third one – the wasting pump (Table 2.2).

Table 2.2 – The main parameters of lab installation

Type of medium	Type of the pump	Pump function	Rotation speed, rpm	Type of hose
tWAS	Verder Flex Van-tage 3000 P R3I EU	Feeding pump	20 and 35	9.5x3.2 mm Tygoon E-3603
Pre-digested tWAS from TPAD1 to TPAD2		TPAD pump	30	8.0x3.2 mm Tygoon E-3603
digested sludge		Wasting pump	3	8.0x3.2 mm Tygoon E-3603

The withdrawn digested sludge was taken for all sorts of analyses executed (Table 2.3).

Table 2.3 – The list and schedule of analyses conducted within all experimental phases

week day	type of analysis	frequency (per month)	tested sample type
Monday	NH_4^+	once	all AD systems (TAD, MAD, TPAD1, TPAD2): digested sludges + substrate
Tuesday	GC, VFA, tCOD, sCOD	four	
Wednesday	TS, TSS, VS, VSS, DS, VDS (1st part)	four	
Thursday	TS, TSS, VS, VSS, DS, VDS (2nd part)		
Friday	GC, VFA	four	

All pumps were regularly (at least, once per month and always when applying a newly sampled tWAS from WWTP) calibrated in triplicate in order to avoid any mistake and confirm proper HRT. At higher extent, it was done due to the fact that all above mentioned types of sludges are of non-Newtonian nature, and very viscous.
At first, the digested sludge was withdrawn from TPAD2, then TPAD pump put pre-digested sludge from TPAD1 to TPAD2, then the fermenter TPAD1 was fed by running the feeding pump. After that, the digested sludge was withdrawn from the single-stage MAD reactor, and immediately after that, it was fed. Finally, the same procedure followed with withdrawing and feeding for TAD reactor. The whole cycle took around thirty minutes and happened once per twenty four hours (semi-continuous model of reactor feeding).

2.1.6 Experimental procedures

As during the whole period of experiments all studied AD systems were tested for clearly defined aims, it was decided to split the overall experimental time into phases. Each phase took, at least, three months in order to fulfil the requirement of minimal

experiment duration a three-time HRT period and provide the stable operation of AD reactors within the analytical step of the experiments.

Hence, the project was divided into four phases (Phases A-D) during each of which certain experiments were held (Table 2.4).

Table 2.4 – Experimental phase division (named in chronological order)

Phase	HRT of a single-stage system, days	HRT of a double-stage system, days	Mixing system*	Mixing speed in a single-stage system, rpm	Mixing speed in a double-stage system, rpm	Reactor insulation
Phase A	13.5	2 / 11.5	N1**	110±1	95 ±1 / 110±1	-
Phase B	13.5	2 / 11.5	N2**	50±1	30±1 / 50±1	-
Phase C	19.0	2 / 17.0	N2**	50±1	30±1 / 50±1	-
Phase D	13.5	2 / 11.5	N1 + N2**	50±1	30±1 / 50±1	+***

Note: *The mixing system N1 corresponds to one-blade impeller; mixing system N2 – to two-blade impeller.

**Mixing system N1 was inside TPAD1; N2 was inside TAD, MAD, TPAD2.

***The insulation consisted of two layers of dense paper and one layer of polyethylene material.

Thus, the mixing system N1 represents a small horizontal paddle (15.0x4.0 cm for T, M, TPAD2, 12.0x4.8 cm for TPAD1). The mixing system N2 consists of the same small horizontal paddle and an added vertical paddle (10.0x15.0 cm for TAD, MAD, TPAD2, 8.2x12.0 cm for TPAD1). All reactor types and their sizes can be found in the Appendix A (Figures A.1.1 and A.1.2).

A full depiction of the averaged working parameters of all AD systems is presented in the Table 2.5.

Table 2.5 – The averaged operational parameters of all AD systems

working parameters	TAD	MAD	TPAD1	TPAD2
Phases A, B, D				
V working, L	8.45	8.45	1.45	8.45
HRT, days	13.4	13.4	2.0	11.6
substrate dose, g/d	630±15	630±15	730±15	
Phase A				
OLR, gCOD/gVSS*d	0.25	0.23	0.19	
VLR, gVS/L*d	3.69	3.69	3.65	
inoculum VS, g/L	31.6±3.9	33.4±1.6	37.5±3.2	30.4±2.0

substrate, VS, g/L		49.5±0.9		
substrate, COD, g/L		71.7±3.6		
Phase B				
OLR, gCOD/gVSS*d	0.26	0.24	0.21	
VLR, gVS/L*d	3.59	3.59	3.55	
inoculum VS, g/L	32.6±0.9	32.0±0.7	35.6±1.0	30.1±0.5
substrate, VS, g/L		48.1±0.6		
substrate, COD, g/L		75.1±3.2		
Phase D				
OLR, gCOD/gVSS*d	0.24	0.22	0.18	
VLR, gVS/L*d	3.62	3.62	3.58	
inoculum VS, g/L	32.9±0.6	32.2±0.6	37.2±2.0	30.4±0.6
substrate, VS, g/L		48.5±1.6		
substrate, COD, g/L		68.2±2.3		
Phase C				
V working, mL	8.45	8.45	1.0	8.45
HRT, days	18.8	18.8	2.0	16.9
substrate dose, g/d	450±15	450±15	500±15	
OLR, gCOD/gVSS*d	0.16	0.14	0.12	
VLR, gVS/L*d	2.25	2.25	2.24	
inoculum VS, g/L	30.7±0.6	31.3±0.5	35.4±1.1	28.6±0.4
substrate, VS, g/L		42.3±0.6		
substrate, COD, g/L		64.1±3.3		

2.2 Analytical Tests: Description and Significance

2.2.1 Biogas production and composition

Biogas represents the gaseous product of the anaerobic digestion and results from the conversion of carbon to methane and carbon dioxide, nitrogen to ammonia and sulfur to hydrogen sulfide. This energy-rich end product usually contains 40-75% methane, 25-60% carbon dioxide and minorities of hydrogen sulphide (0.002-5%), ammonia (<1%), oxygen (0-1%) and trace of volatile organic compounds (Franco-Morgado *et al.*, 2021). The biogas has LCV of around 20-25 MJ/Nm^3 based on the methane content (Fu *et al.*, 2020). Therefore, all the energetic balances were provided in terms of methane. Biogas production was measured by gas meter Ritter MilliGascounter "MGC-1 V3.4 PMMA" (Q_{min} = 1 mL/h, Q_{max} = 1 L/h, p_{max}: 5.0 mbar) from "Ritter", Germany. The Milli gascounters were filled with the HCl 1.8%-solution as the liquid phase to avoid any dissolving and outgassing process (mainly this relates to the presence of CO_2) to the greatest possible extend.

The biogas composition was assessed using the gas chromatograph (GC) Shimadzu GC-2014 ("Shimadzu Corporation", Japan) with a thermal conductivity detector (temperature 185 °C) and injection via on-column with packed column (packed by Hayesep D 100/120 mash; oven: isotherm 130 °C, flow 30ml/min, carrier gas – Helium). 1.0 mL of biogas produced was withdrawn with a tight syringe, and introduced into the column, which evaluated the gaseous composition. The

percentage of carbon dioxide, methane and nitrogen was detected in each sample. Hydrogen content was monitored using GC 8000 Top Gas Chromatograph by "CE Instruments", United Kingdom with a thermal conductivity detector (temperature 185 °C) and injection via on-column with packed column (packed by Hayesep D 100/120 mash; oven: isotherm 100 °C, flow 30ml/min, carrier gas – Helium).

The specific methane production was calculated in the following way:

$$Qsp.methane = \frac{!''^{\#\$\%\&'\#}}{\bigcirc *+,\#*./O,12,\$3\&\$\#)}, \text{ L/g COD}^{\text{added}}, \quad (1)$$

where $Q_{methane}$ – daily methane production, L/day;
W_{dose} – substrate volume added, L/day;
$COD_{substrate}$ – COD concentration in the substrate, g COD_{added}/L.

2.2.2 Biochemical Methane Potential tests

The progressive acceptance of AD to treat complex organic wastes called upon the need for an experimental tool to study and detect digestion biokinetic constants and measure biochemical methane potential (BMP) of different substrates. The Biomethane Potential Assay is a well-established method and was developed with the intention to establish the final methane yield of organic substances, the potential rate and extent of transformation of biomass and wastes to methane and for monitoring anaerobic toxicity (Gunaseelan, 1997). The BMP test which expresses an index for anaerobic biodegradation potential is conducted in several batches under defined conditions at which the amount of biomethane generated by mixture of organic substrate and anaerobic inoculum is assessed. Different techniques can be adopted to measure the biogas production and the most frequently used are volumetric and manometric methods (Giovanni *et al.*, 2012). The BMP is expressed as the volume of dry methane gas under standard conditions (273.15K and 101.33kPa) per mass of volatile solids added. The unit of BMP is NL_{CH4}/kg_{VS} (Holliger *et al.*, 2016).

Giovanni *et al.* (2012) state that BMP assay is a time demanding method and its operation goes up to 30 days. However, many commend this test for being rather facile, repeatable and reliable (Giovanni *et al.*, 2012; Gunaseelan, 1997).

Agreed comprehensive international guidelines by Holliger *et al.* (2016) coordinate testing in laboratories around the world, allowing the scientific community to share and discuss results with confidence.

The BMP tests were held according to the protocol by Holliger *et al.* (2016).

The main aim of BMP testing during the experiments in this study was to examine not onlt biodegradable efficiency, but also biomethane potential and specific methanogenic activity (SMA) at two different HTRs and correlate it to the quality of the digested sludge from each studied AD reactor.

The peak of SMA for each test, expressed in gCOD/gVSS/d was defined on the second-third day of the experiment according to the steepest slope of the methane production curve with the measurement frequency of maximum eight hours during the first six days of the test.

In all experiments, the mesophilic and thermophilic inocula were collected from the correspondent laboratory anaerobic digesters. As a substrate was used the same tWAS applied as a substrate to the laboratory digesters.

Glass bottles with a volume of 120 mL were working volume was 80 mL with the remaining 40 mL left for gas space. Inoculating thermophilic and mesophilic inocula is pipetted from a beaker containing the mixed and homogenized sample and poured into glass bottles. Then the substrate is added to the inoculum. Blank samples without any substrate were also prepared to check the gas production of inoculum. pH was measured after 80mL of slurry has been poured, and the bottles were sealed tightly with rubber stops and ring caps. Afterwards, each bottle was flashed with nitrogen gas for 2.5 minutes in order to remove all oxygen and create anaerobic conditions. Prepared bottles were then kept in their respective thermophilic and mesophilic chambers for further observation.

Temperature of 38.0±1.0 ^{0}C and 57.0±1.0 ^{0}C, respectively, for mesophilic and thermophilic conditions, was maintained continiously in thermo room and thermobox.

Biogas quality and composition are obtained using gas chromatography (GC-2014 Shimadzu) with thermal conductivity detection. And biogas yield, or the volumetric biomethane is determined using a volume displacement system with communicating vessels.

Each BMP test was conducted for over twenty days until either there was no biomethane production, the daily biomethane production was lesser than 1.0% of the accumulated volume of methane, or both.

2.2.3 Suspended solids

The solid analysis of the sludge forms the basic characterization of the sample. The test determines the content of Total Solids (TS), Volatile Solids (VS), Dissolved Solids

(DS), Fixed Solids (FS), Total Suspended Solids (TSS) and Volatile Suspended Solids (VSS).

In order to determine the solid content of the sludge, standard procedures described by APHA (The American Public Health Association, 2012) were used. Sludge to be tested is first manually mixed so that all contents are homogenized. The sample is then transferred into a 100-150 mL beaker and placed on a magnetic stirrer and mixed for proper homogenization. The sample is continuously mixed until sub-samples for all analytical tests have been taken. Rectangular pieces of aluminum foil are molded into cups and labelled respectively according to the sample they will hold. Their weight is observed and recorded using an analytical balance Sartorius MC1. 15mL samples are then pipetted and transferred to respective aluminum dishes. To determine the TS content, dishes are dried using a Binder 105 ^{0}C oven at (105±2) ^{0}C for approximately twenty four hours, so that all the water in the sample is completely evaporated and the dish is then weighed to constant weight after cooling in desiccators. Further, to obtain the VS content in the sample, the dishes are firmly folded to avoid sample loss and placed in a furnace „Technologie THP 48 furnace & oven" to be heated at a temperature of 550 ^{0}C for two hours until the constant weight of a sample is reached. At 550 ^{0}C, the organic matter in the sample undergoes complete mineralization along with which some insignificant inorganic matter also volatilizes. The dishes are then cooled in desiccators and weighed. The analytical balance is connected to computer wherein all values are recorded on an excel sheet. The TSS is then obtained by calculating the difference between the total and dissolved solids in the sample. The residue obtained after drying at 550 ^{0}C is termed as FS which represents the inorganic content in the sample.

2.2.4 Chemical Oxygen Demand

The standard test for Chemical Oxygen Demand (COD) is based on the oxidation of organic material wherein the consumed amount of oxygen represents the COD parameter value. At a high temperature (150 ^{0}C) with the aid of an oxidizing medium, complete mineralization occurs and most of the pollutants are converted to CO_2 and H_2O.

To determine the total COD (tCOD) samples are usually diluted so that measured COD values fall within the detection limits of the spectrophotometer Hach Lange DRB-3900 (set at 600 nm wavelength). 5mL of the sample is pipetted and transferred into a standard flask of 100 mL. The flask is filled with distilled water and shaken. The solution is then poured into a beaker and placed on a magnetic stirrer to mix and homogenize a sample. If the sample is still very polluted, then the dilution procedure is being repeated until it is acceptable – visually transparent. 2.5 mL of the diluted sample is pipetted and poured into digesting glass test tubes which hold 1.5 mL of oxidizing reagent potassium dichromate. 3.5mL of catalyzing reagent sulfuric acid is

added and the culture tubes are capped tightly. The test tubes are then boiled for 2 hours in an incubating mineralizer Hach Lange DRB-200 at 105 ^{0}C. After mineralization, test tubes are cooled to room temperature. A blank sample with distilled water is prepared to calibrate and test the spectrometer. All tubes are then cleaned with ethanol to remove any surface particles that may interfere with the spectrometry. COD is noted using absorbance at wavelength of 600 nanometer in the spectrophotometer which is based on the change in colour of potassium dichromate. All samples are measured in triplicates.

To determine the soluble COD (sCOD) samples are perfomed in a similar way excluding several dilution steps. Normally, it was required to dilute just once in order to be able to use spectrophotometer HACH LANGE DRB-3900 set at 600 nm.

2.2.5 Ammonia nitrogen

Among all the chemicals that anaerobic digestion is vulnerable to, ammonia is probably the most significant one. Ammonia is usually formed as a result of the degradation of proteins and other nitrogen-rich compounds. Even though its presence is necessary for bacterial growth and system buffering, a consistent increase of total nitrogen ammonia (TAN) could be fatal for the microorganisms.

Ammonia can be found both as ions ammonium (NH_4^+) and free ammonia (NH_3), and their relative concentrations depend on pH and temperature. Specifically, with an increase in those two parameters, the balance is shifted in favor of NH_3.

Total ammonia nitrogen (TAN) concentrations (mg NH_4-N/L) were investigated using a distillation unit K-360 Büchi. At first, 10 mL of the sample was diluted with distilled water, then, mixed with sodium hydroxide in order to convert all ammonium (NH_4^+) into ammonia (NH_3) and distilled employing the K-360 distillation device. After condensation, the liquid distillate was collected in a flask with boric acid (H_3BO_3) and titrated with sulfuric acid (H_2SO_4) until intensive pink colour appears. Lastly, TAN concentration was calculated by measuring the acid consumption. The measurements were performed in technical duplicates.

After the ammonia nitrogen concentration was measured, it was recalculated into free ammonia (FA) concentration accoring *et al* to Anthonisen (1976):

$$FAconc. = TANconc. * 10^{56} / \exp\left(\frac{7899}{(:;:'')}\right) + 10^{56}, \quad (2)$$

where TAN conc. – measured total ammonia nitrogen, mg/L;
pH – pH of the medium measured at the time of TAN measurement;
$\exp\left(\frac{7899}{(:;:'')}\right)$ – ammonia rate constant according to Anthonisen *et al.* (1976);

T = 273K;
T – temperature at the time of TAN measurement, ºC.

2.2.6 pH

The increased biogas yield was accompanied by an increase in pH for both mesophilic and thermophilic sludge. Kim *et al.* (2002) suggest that increased biogas yield is usually observed alongside an increase in pH as well because the methanogens reduce the concentration of volatile fatty acids (VFAs) during methanogenesis and increase alkalinity. Amani *et al.* (2011) also noted a direct relation between increasing pH and biogas yield and explained that usually there could be a decrease in pH and lesser growth of anaerobes due to presence of VFAs in anaerobic digestion, which inhibit methanogenesis. The increase in pH accompanied by increasing biogas yield implies an efficient destruction of VFAs and flourishing methanogenic activity.

pH was measured by four probes Polilyte Plus H Arc 225 ("Hamilton Bonaduz AG", Switzerland) connected to the computer and LabView 2012 software to be able to log the data in online. The same source of controlled temperature was used.

2.2.7 VFA

Volatile Fatty Acids (VFA) exhibit a quite significant inhibiting action. Firstly, they can cause toxic outcomes on methanogens archaea at a concentration of 6.7 to 9.0 mol/m^3. Furthermore, they can also lower the pH, which will drive to an additional inhibition of methanogenic activity.

VFAs were measured weekly employing GC Shimadzu GC-2010 ("Shimadzu corporation", Japan) with a flame ionization detector and capillary column "CP-Vax58" (25 m lenght and 0.25mm inner diameter). The oven program was the following: 70 °C with a rate of 15 °C/min to 134 °C and isoterm for 1 min. Totat time of analysis was 5.27 min. Injection temperature was 270 °C at the split mode. Detector temperature was 300 °C.. The samples were prepared by centrifuging the digested sludge for 10 minutes at 13083 rpm (centrifuge Sigma 3 – 16 P, "Sigma Laborzentrifugen GmbH", Germany), filtered through a filter ACRODISC PSF, ("Pall Corporation", the USA) with 0.45 μm diameter pore size and diluted ten times before the measurement.

The VFA concentration was calculated in the following way:

$$QVFA = \frac{.<=>*<3\#\&?\$+3}{\text{)*+,\#*./O,12,\$3\&\$\#}}, \text{ g/gCOD}_{\text{added}} \quad (3)$$

where C_{VFA} – daily VFA concentration, g/L;

$V_{reactor}$ – working volume of the reactor, L;
W_{dose} – substrate volume added, L/day;
$COD_{substrate}$ – COD concentration in the substrate, g COD_{added}/L.

2.2.8 Digested sludge dewaterability

The sludge quality, namely, digested sludge dewaterability of the three types of AD, was evaluated by two methods:

a) separation via centrifugation;
b) filtration and compression via mechanical pressing.

In addition, the elemental analysis of sludge was performed in order to calculate two parameters: the lower calorific value of sludge and an universal factor recommended for an estimation of the sludge cake TSS concentration in the full-scale AD (Svennevik *et al.*, 2019), so-called *C/N*ash parameter*, for conventional AD:

$$5.53 * {}^{C}\mathrm{A}_{N} * ash + 7.14, \quad (4)$$

where *C/N* – a ratio of C and N content in sludge;
ash – the mass fraction equal to (1-VSS/TSS);
5.53 and 7.14 – the empirical values obtained experimentally.
Finally, the efficiency of hygienization of digested sludge was evaluated by indicator pathogenic bacteria assessment.

2.2.8.1 Dewatering via centrifugation

The key point of this method is measuring the cake concentration after centrifugation of digested sludge in lab scale analytical centrifuge.

For this method, the centrifuge Sigma 3 – 16 P ("Sigma Laborzentrifugen GmbH", Germany), with the relative centrifugal force G = 15309, was used. At 13083 rpm for ten minutes all samples were centrifuged, the weight of separated digested sludge was measured. The higher weight – the better quality of the digested sludge. It is important to mention that separation via centrifugation is considered to be a method that the most closely describes the dewatering qualities of the sludge in comparison to the real full-scale one. All sludge samples were centrifuged, the weight of separated digested sludge samples was measured. Meanwhile, the amount of TS in non-separated digested sludge was measured. Then, cake concentration was calculated as a ratio of TS in digested sludge and a weight of a separated digested sludge sample. As the supernatant was not transparent, hence, it was taken in consideration, and TS were evaluated in the reject water as well. It is also important to mention that no flocculants were used to characterize natural properties of sludge.

To be able to assess the dewatering ability of the digested sludge, we calculated the sludge cake concentration and introduced it as dewaterability coefficient (D), %. It was calculated as follows:

$$Dewaterability\ coefficient\ (D) = (Wtotal\ solids)\ /\ (Wsludge\ sample) * 100\%, \quad (5)$$

where: $W_{total\ solids}$ – the weight of total solids of digested sludge sample after centrifugation, g;
$W_{sludge\ sample}$ – the weight of digested sludge sample after centrifugation, g.

2.2.8.2 Mechanical pressing

This method if based on usage of a specific commercial equipment – mini-press Mareco MMP-3/2 ("Amfitech", the Netherlands) (Figure 2.1).

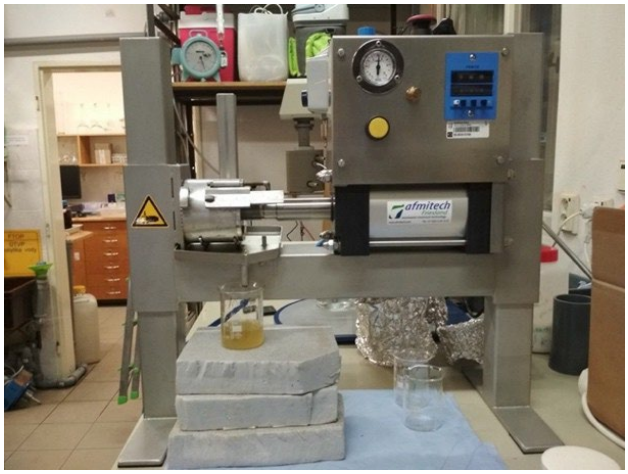
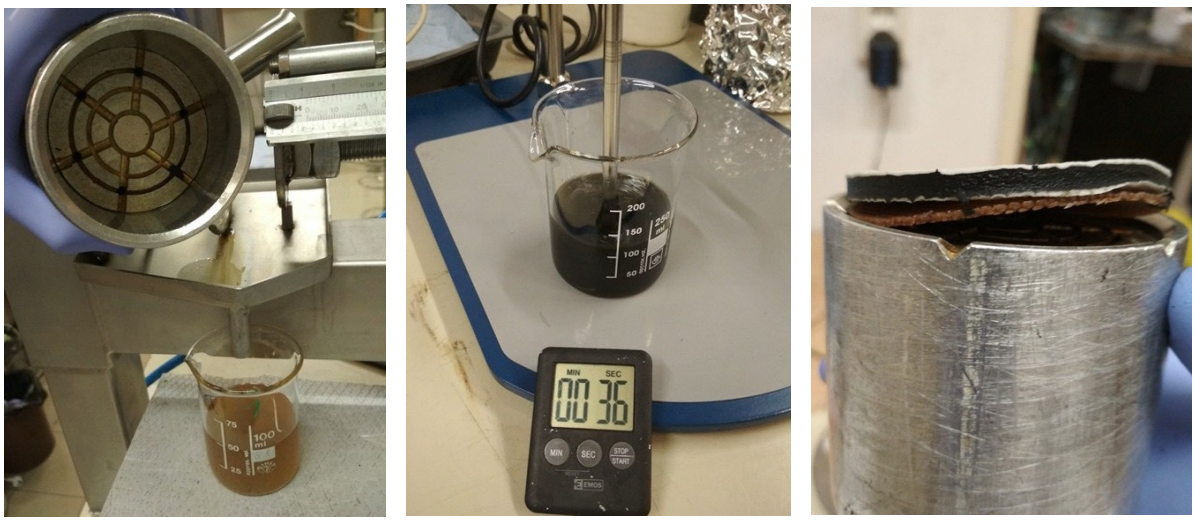


Figure 2.1 – The laboratory mini-press Mareco

As the first step, TS of the digested sludge samples from every reactor were determined. As the second step, one-liter solution of concentrated polymer Superfloc C-494HMW ("Kemira", Finland) was prepared with a concentration of 5.0 g/L. The solution was always fresh and prepared right before (at maximum, four hours) the experiment and was used between four and eight hours after its preparation. Tap water was used to prepare the suspension. Then, the suspension was mixed thoroughly at 1000 rpm by blade impeller until no lumps are observed in the whole volume of the transparent solution. That process lasted up to four hours before its usage to make sure it is ready for the experiments. Then, based on the TS concentration of the tested sludge, the calculated correspondent flocculant solution was added to 50 mL of every type of digested sludge. That amount of flocculant stock solution varied from 18 to 23 mL depending on exact value of TS and type of every digested sludge and was obtained during the pre-liminary experiments. After that, a digested sludge sample together with dose of flocculant solution was mixed at 700 rpm during three-minute period of time (Figure 2.2).



(a) (b) (c)

Figure 2.2 (a-c) – Elements of dewatering process: a – a jar for a mixture of a digested sludge sample with flocculant dose; b – a mixing process at 700 rpm within three minutes; c – digested sludge sample after the dewatering process run in mini-press Mareco

The intensity of mixing a sample together with flocculant solution dose was chosen experimentally in order to provide flocs' formation and avoid flocs' destruction. During the whole experimental period was used the same commercial flocculant.

The dewatering process was run under five bar and lasted 999 seconds (a full cycle of mini-press Mareco). There was used filter paper Filtrak (No.388, diameter 7 cm; 0.015 mg), soft and wide pores, fast filtering for coarse deposits. The supernatant was collected (Figure 2.2) and weighted on the calibrated analytical balance Acculab ALC-3100.2. The quality of supernatant in terms of TSS was measured and its cleanness was assessed every time.

2.2.8.3 Elemental analysis and lower calorific value calculation

For the Elemental Analysis (EA), an integrated sample was collected for each type of digested sludge within four days in a row. It was taken in a folie dish and dried in the oven at 105 °C until there is enough amount ash for EA (at least, one gram). EA was performed with a help of Elementar vario EL Cube ("Elementar Analysensysteme GmbH", Germany).

For each phase, the EA was performed in triplicate with an interval of two weeks. The Lower Calorific Value (LCV) was calculated based on averaged data from EA of digested sludge samples' ash content for both phases.

Thus, LCV, $kJ.kg^{-1}$ was calculated according to the following formula (Nzihou *et al.*, 2014) – Equation 6:

$$LCV = 4.18 * (94.19 * C - 0.5501 - 52.14 * H), \quad (6)$$

where 4.18, 92.19, 0.5501, 52.14 – empirical coefficients calculated experimentally;
C – carbon;
H – hydrogen.

2.2.9 Microbiological analyses

2.2.9.1 Microbiological tests for pathogenic activity of the digested sludge

The pre-treatment of digested sludge samples was performed in a following way. One gram of each sample (very well shacked) was put into 9 mL of physiological solution (9g of NaCl in 1L of distilled water and sterilised).

For cultivation, the solution of sludge and physiological solution was diluted to 10^{-2} and 10^{-3}. To measure the total counts of bacteria, as indicators of organotrophic and faecal contamination were chosen the culturable aerobic microorganisms cultivated at 22 °C and 36 °C (EN ISO 6222, used medium Tryptone Yeast Extract Agar;), total coliforms and *E. coli* (Czech standard norms, ČSN 75 7837 and used medium Endoagar, ČSN 75 7835 and used medium M-FC Agar Base) and *Clostridium perfringens* (Council Directive 98/83/EC in Directive No. 252/2004 Coll., M-CP medium). The cultivation procedure for microorganisms cultivated at 22 °C and 36 °C was as described below. 1 mL of the pre-treated and diluted digested sludge sample was put to a Petri dish, then, the correspondent sterilised growth medium was poured into the Petri dish. The procedure of the faecal contamination indicators cultivation was slightly different: 0.2 mL of the pre-treated and diluted digested sludge sample was placed directly on the surface of the correspondent sterilised growth medium put in a Petri dish earlier.

2.2.9.2 Sampling for biological analyses (FISH and DNA extraction)

The grabbed samples in volume of 1-2 mL were taken during the Phase B and collected in sterile 2 mL eppendorfs (“Eppendorf AG”, Germany), fixed with equal volumes of ethanol and frozen.

For Fluorescence in situ hybridization (FISH), there were three samples taken from each reactor four times per tested period from the levels correspondent to three reactor valves: low, middle and high (Supplementary material, Appendix A, Figures A.1.1. and A.1.2, Appendix B, Table B.1.1).

For DNA extraction, there were three samples in total taken from: the central part of the reactor TPAD1 (D1), the wall of the reactor TPAD1 (D2), the central part of the reactor TPAD2 (D3) (Supplementary material, Appendix B, Table B.1.2).

2.2.9.3 FISH

FISH analyses were performed according to Niesen (2009) on slides or in suspension (WET-FISH for the samples from thermophilic digested sludge sampled on 13.05.2019 and 10.08.2019). Hybridization buffer (8 µl for FISH on slides or 16 µl for WET-FISH) with addition of 50 ng/µl EUBmix oligoprobe (equimolar mixture of EUB338, EUB338II and EUB338III, (Loy, 2003) labelled with FLUOS Green (excitation 494 nm, emission 523 nm) for the bacteria and ARCH915 (ARC915; by Alm *et al.*, 1996) labelled with Cy3 (excitation 552 nm, emission 565 nm) for archaea. Overnight incubation at 46°C and washing were performed on slides or in suspension in 1.5 mL eppendorfs. Samples from thermophilic digested sludge sampled on 13.05.2019 and 10.08.2019 needed special treatment as the biomass did not want to stick to the slides. After failed standard, FISH analyses were done again. Then the WET-FISH procedure was performed.

2.2.9.4 DNA extraction

DNA was extracted according to manufacturer instructions with the FastDNA™ Spin Kit for Soils (MP Biomedicals, Santa Ana, USA). The concentration and purity of DNA were measured by a nano spectrophotometer (BioDrop µLITE, Biodrop, UK).

2.2.9.5 Library preparation

16S rRNA gene region V4 sequencing libraries were processed by external contractor - DNA Sense, based on an Illumina protocol (2015). PCR reaction (25 L, using up to 10 ng of extracted DNA as template) were done in duplicates, which were pooled after PCR. Each reaction contained (12.5 L) PCRBIO Ultra mix (PCR Biosystems, USA) and 400 nM of each forward and reverse tailed primer mix. PCR was conducted as follows: Initial denaturation for 2 min at 95 °C for, 30 cycles of amplification (95 °C for 15 s, 55 °C for 15 s, 72 °C for 50 s) and a final elongation at 72 °C for 5 min.

The primers were designed according to Illumina protocol (2015) and contain primers targeting the Archaea and Bacteria, 16S rRNA gene region V4: [515FB] GTGYCAGCMGCCGCGGTAA and [806RB] GGACTACNVGGGTWTCTAAT (Apprill *et al.*, 2015). The primer tails enable attachment of Illumina Nextera adaptors necessary for sequencing in a subsequent PCR. The libraries were purified with Agencourt Ampure XP Beads (Beckman Coulter, USA) with a bead to sample ratio of 4:5. DNA was eluted in 25 L of nuclease free water (Qiagen, Germany). DNA concentration was measured using Qubit dsDNA HS Assay kit (Thermo Fisher Scientific, USA). Gel electrophoresis using Tapestation 2200 and D1000/High sensitivity D1000 screentapes (Agilent, USA) was used to validate product size and purity of a subset of sequencing libraries. Sequencing libraries were prepared from the purified amplicon libraries using a second PCR. Each PCR reaction (25 L)

contained PCRBIO HiFi buffer (1x), PCRBIO HiFi Polymerase (1 U/reaction) (PCRBiosystems, UK), adaptor mix (400 nM of each forward and reverse) and up to 10 ng of amplicon library template. PCR was conducted with the following program: Initial denaturation at 95 °C for 2 min, 8 cycles of amplification (95 °C for 20 s, 55 °C for 30 s, 72 °C for 60 s) and a final elongation at 72 °C for 5 min. The resulting sequencing libraries were purified using the standard protocol for Agencourt Ampure XP Beads (Beckman Coulter, USA) with a bead to sample ratio of 4:5. DNA was eluted in 25 L of nuclease free water (Qiagen, Germany). DNA concentration was measured using Qubit dsDNA HS Assay kit (Thermo Fisher Scientific, USA). Gel electrophoresis using Tapestation 2200 and D1000/High sensitivity D1000 screentapes (Agilent, USA) was used to validate product size and purity of a subset of sequencing libraries.

2.2.9.6 DNA sequencing

The purified sequencing libraries were pooled in equimolar concentrations and diluted to 2 nM. The samples were paired-end sequenced (2x300 bp) on a MiSeq (Illumina, USA) using a MiSeq Reagent kit v3 (Illumina, USA) following the standard guidelines for preparing and loading samples on the MiSeq. >10% PhiX control library was spiked in to overcome low complexity issues often observed with amplicon samples.

Bioinformatic processing Forward and reverse reads were trimmed for quality using Trimmomatic v. 0.32 (by Bolger *et al.*, 2014) with the settings SLIDINGWINDOW:5:3 and MINLEN: 225. The trimmed forward and reverse reads were merged using FLASH v. 1.2.7 (by Magoc & Salzberg, 2011) with the settings -m 10 -M 250. The trimmed reads were dereplicated and formatted for use in the UPARSE workflow (Edgar, 2013). The dereplicated reads were clustered, using the usearch v. 7.0.1090 -cluster_otus command with default settings. OTU abundances were estimated using the usearch v. 7.0.1090 -usearch_global command with -id 0.97 -maxaccepts 0 -maxrejects 0. Taxonomy was assigned using the RDP classifier (Wang *et al.*, 2007) as implemented in parallel_assign_taxonomy_rdp.py script in QIIME (Caporaso *et al.*, 2010), using –confidence 0.8 and the MiDAS database v. 1.23 (McIlroy *et al.*, 2017), which is a curated database based on the SILVA database, release 123 (Quast *et al.*, 2012). The results were analysed in R v. 3.5.2 (by R Foundation for Statistical Computing, 2010) through the Rstudio IDE using the ampvis package v.2.4.5 (Albertsen *et al.*, 2015).

2.3 Digester modelling

2.3.1 Simulation of the flow field in the used vessels

To understand the impact of mixing on the flow field in the used vessels Computational Fluid Dynamic (CFD) program Ansys Fluent 19.2 was used. Geometry of the vessels was built in a Design Modeller program (part of Ansys Workbench

bundle) with consequent meshing of the computational domain. Due to high gradient of the velocity, the number of elements used to solve mass and momentum equations was for larger vessel around 1.2 million while for smaller vessel it warries from 1.3 to 2.2 million. Due to high viscosity of the sludge the flow was model using laminar model. To capture the measured dependency of the sludge viscosity as a function of the applied shear rate this was measured using rotational rheometer RC20 using temperature of 28°C. The density of the sludge was considered to be equal to 1000 kg/m^3, slightly higher than water at 28 °C to compensate the presence of solid particles in the sludge. Pressure-velocity coupling was resolved by SIMPLE scheme while pressure and momentum spatial resolution was discretized using Second order and Second order upwing scheme, respectively. The simulations were considered as converged when the residuals for all modelled quantities reached values 1*10^{-5}.

2.4 Statistics

2.4.1 ANOVA technique and Scheffé method

For performing the statistical analysis, statistical technique ANOVA (Analysis of Variance) was used. ANOVA is the well-known statistical technique used to check if the average values of two or more parameters are significantly different from each other, having two or more categories. The parameters in this study are different technological procedures with repetitions which have been done in different time. There were three technological procedures: methane production and dewaterability measured by two different methods.

A one-way ANOVA technique was applied. That meant that only one independent variable – the temperature of AD process – was used. Statistical verification of significance was done at significance level $a = 0.05$. For statistically significant results, the further Scheffé method was applied.

The Scheffé method was used for the multiple comparison of the average values (or contrasts). The estimation of each contrast for three procedures was defined as follows (according to Equation 7):

$$\psi_{i,j} = \bar{x}_i - \bar{x}_j, \tag{7}$$

where $i \neq j$ and are qual from 1 to 3, to the number of contrasts.

The Scheffé test is the most conservative procedure as it provides the narrowest confidence interval. The confidence interval within Scheffé test is defined as (Equation 8):

$$\psi_{i,j} \pm \sqrt{(I-1) * s * F * \left(\frac{c_i}{n_i} + \frac{c_j}{n_j}\right)}, \tag{8}$$

where $\psi^{X}_{@,B}$ is the *i-*, *j-* contrast, *I* is a number of parameter levels (in this case, I = 3), r_i, r_j is a number of repetition in *i-*, *j-* levels, *s* – the residual standard deviation (from ANOVA), F is the critical F-value for (*1-a*) and ((*I-1);* (*N-I*)) degrees of freedom, *N* is the total number of experiments in ANOVA table.
If the confidence interval for *i-*, *j-* contrast contains zero value, the contrast is non-significant.

2.5 Life Cycle Assessment
2.5.1 Goal and scope definition of LCA

The goal of this study was to compare the potential environmental impacts of three types of AD processes: i) mesophilic; ii) thermophilic; iii) TPAD systems.

To this end, two LCA cases with two different functional units (FU) were conducted: first for an activated sludge WWTP with the three different AD systems (here named as WWTP-LCA); and second for the sludge line alone with the three different AD systems (here named as SL-LCA).

Since it has been reported in literature that the choice of the FU may change the overall balance of environmental impacts from harmful to beneficial and vice-versa (Sills *et al.*, 2020), these two FUs were chosen considering the major outputs and functions of a WWTP. It is, for the first case, the FU was 1 m^3 of treated wastewater, as the main function of the WWTP is to treat the wastewater stream. For the second case, the FU was 1 m^3 of produced methane, as one of the main functions in AD systems (also the secondary one within WWTP) is to produce energy out of the methane contained in the biogas. Methane was taken instead of biogas, as biogas might have a different content of methane depending on the AD system.

The two FUs have been coupled with the system expansion approach (the alternative production of energy and fertilizers) and adopted to evaluate the environmental burden of AD at both scales, the whole WWTP (to assess the contribution of the sludge line to the whole WWTP) and the sludge line alone (to highlight the potential environmental benefits from methane production as an additional function of the system beyond the wastewater treatment). The application of two FUs would demonstrate a deeper, more comprehensive and more transparent picture of AD implementation at the sludge line and at the WWTP. Thus, applying the two FUs helps to present the environmental profile of each AD from different points of view (Lamnatou *et al.*, 2019; Sills *et al.*, 2020).

For both LCAs, a period of one-year operation was considered, as it is a timescale that is long enough to assess the averaged operational parameters of any WWTP, including the fact that the construction part was not estimated in the impacts' analysis. The impacts of the construction phase were not accounted for, since the dimensioned

WWTP is the same for all three scenarios and LCAs, and it would make the difference among three AD systems' exploitation less evident (Lamnatou *et al.*, 2019; Timonen *et al.*, 2019).

To sum up, the system's boundaries – for the first and second LCAs – consider the year-around operation of the whole WWTP with three different AD technologies and the sludge line alone with three different AD technologies (Figure 2.5), respectively. Thus, three scenarios were considered in each LCA; namely, TAD, MAD and TPAD.

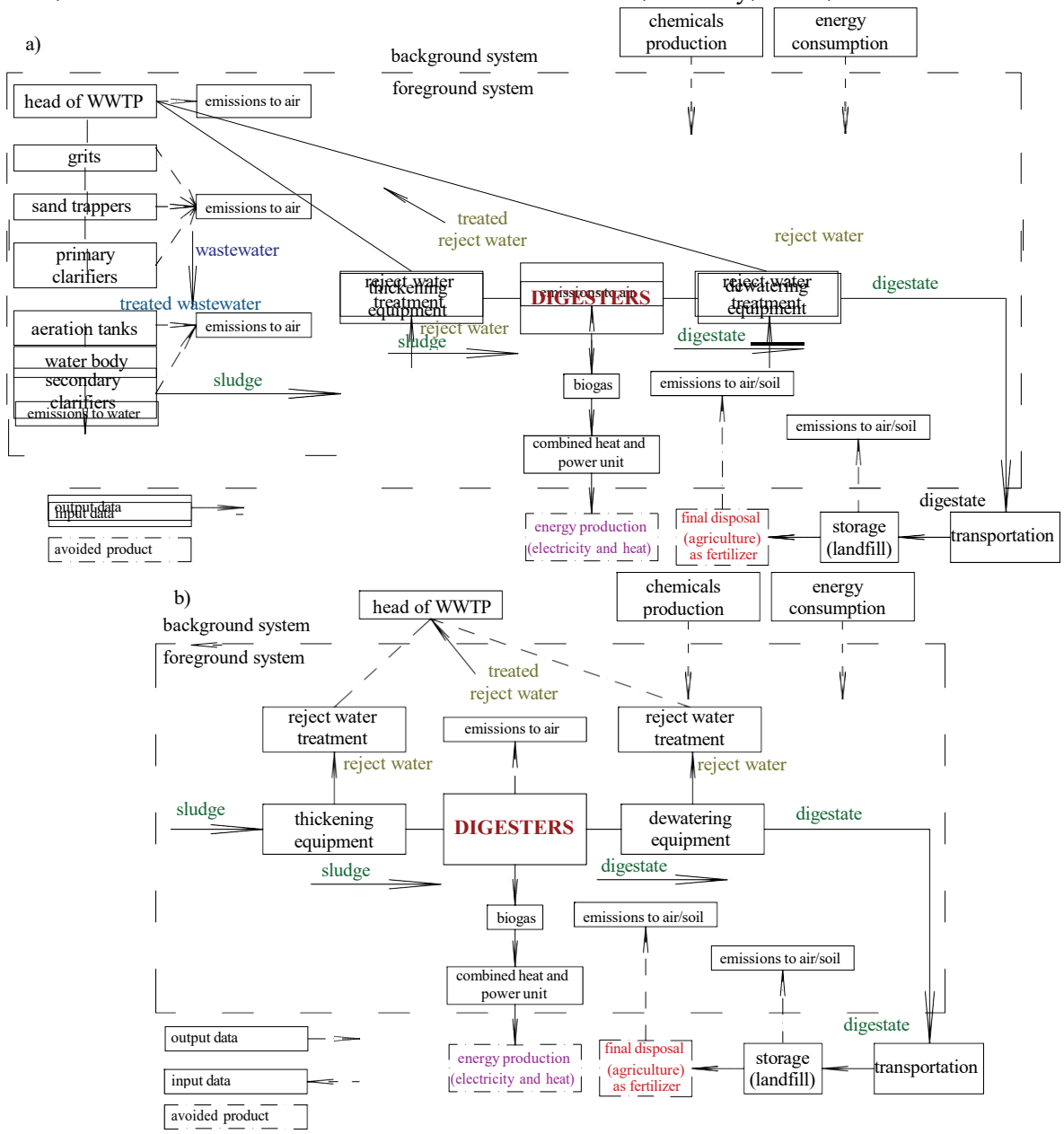


Figure 2.5 (a-b) – Flowcharts: (a) of the whole wastewater treatment plant (WWTP-LCA) and (b) only the sludge line (SL-LCA)

2.5.2 Inventory analysis

Inventory data for the WWTP-LCA and SL-LCA are summarized in Tables 2.6 and 2.7.

Table 2.6 – Inventory data for the whole WWTP (FU: 1 m^3 of treated wastewater)

Type of data	WW/SL	Item	Referred to FU			Unit
			TAD	MAD	TPAD	
Input data						
energy consumption	WW+SL	total energy consumption	0.42	0.39	0.39	kWh/m^3
	WW	only for WW	0.32	0.31	0.3	kWh/m^3
	SL	only for SL	0.1	0.08	0.09	kWh/m^3
reagents	WW	phosphorus precipitation agent	0.07	0.07	0.03	kg/m^3
		sludge anti-bulking agent	0.05	0.05	0.05	kg/m^3
	SL	thickening agent	$1.23 \cdot 10^{-4}$	$1.23 \cdot 10^{-4}$	$1.23 \cdot 10^{-4}$	kg/m^3
		anti-foaming agent	$2.63 \cdot 10^{-4}$	$5.27 \cdot 10^{-4}$	$5.27 \cdot 10^{-4}$	kg/m^3
		dewatering agent	0.002	0.001	0.002	kg/m^3
		chemo dezodoration agent	$1.0 \cdot 10^{-5}$	$1.0 \cdot 10^{-5}$	$1.0 \cdot 10^{-5}$	kg/m^3
		bio dezodoration agent	$2.2 \cdot 10^{-5}$	$2.2 \cdot 10^{-5}$	$2.2 \cdot 10^{-5}$	kg/m^3
Output data						
waste	**WW**	sand	0.02	0.02	0.02	kg/m^3
		sand	0.001	0.001	0.001	t*km/m^3
		coarse waste	0.04	0.04	0.04	kg/m^3
		coarse waste	0.002	0.002	0.002	t*km/m^3
wastewater air emissions		CO	9	9	9	g/m^3
		SO_2	0.03	0.03	0.03	g/m^3
		Ozone	0.08	0.08	0.08	g/m^3
		N_2O	0.05	0.05	0.05	g/m^3
		H_2S	0.0008	0.0008	0.0008	g/m^3
		NH3	2.8	2.8	2.8	g/m^3
		N_2O	1.8	1.81	1.81	g/m^3
		CO_2	81	81	81	g/m^3
		N_2	41	41	41	g/m^3
wastewater contaminants		COD	0.04	0.04	0.04	kg/m^3
		SS	0.009	0.009	0.009	kg/m^3
		TN	0.02	0.02	0.02	kg/m^3
		TP	$6.7 \cdot 10^{-4}$	$6.7 \cdot 10^{-4}$	$6.7 \cdot 10^{-4}$	kg/m^3
		As	$9.6 \cdot 10^{-6}$	$9.6 \cdot 10^{-6}$	$9.6 \cdot 10^{-6}$	kg/m^3
		Pb	$1.5 \cdot 10^{-5}$	$1.5 \cdot 10^{-5}$	$1.5 \cdot 10^{-5}$	kg/m^3

		Cd	$1.2 \cdot 10^{-6}$	$1.2 \cdot 10^{-6}$	$1.2 \cdot 10^{-6}$	kg/m³
		Cr	$2.2 \cdot 10^{-6}$	$2.2 \cdot 10^{-6}$	$2.2 \cdot 10^{-6}$	kg/m³
		Cu	$2.9 \cdot 10^{-5}$	$2.9 \cdot 10^{-5}$	$2.9 \cdot 10^{-5}$	kg/m³
		Ni	$6.9 \cdot 10^{-6}$	$6.9 \cdot 10^{-6}$	$6.9 \cdot 10^{-6}$	kg/m³
		Zn	$3.2 \cdot 10^{-5}$	$3.2 \cdot 10^{-5}$	$3.2 \cdot 10^{-5}$	kg/m³
		Hg	$0.2 \cdot 10^{-7}$	$0.2 \cdot 10^{-7}$	$0.2 \cdot 10^{-7}$	kg/m³
digested sludge contaminants	**SL**	dewatered digested sludge (wet)	0.82	0.86	0.78	kg/m³
		dewatered digested sludge (wet)	0.032	0.034	0.031	t*km/m³
avoided products		electricity	0.23	0.22	0.35	kWh/m³
		heat	0.26	0.25	0.39	kWh/m³
air emissions		H_2S	$2.0 \cdot 10^{-4}$	$2.0 \cdot 10^{-4}$	$2.0 \cdot 10^{-4}$	g/m³
		CH_4	0.02	0.02	0.02	g/m³
		CO_2, biogenic	28	28	28	g/m³
		CO_2, not biogenic	44	44	44	g/m³

Note: *For TAD and TPAD, the transportation distance was 40 km to the sludge handling plant.

**According to the microbiological analyses and other sources (Akgul *et al.*, 2017), TPAD and TAD digested sludges meet the requirements of Class A biosolids. In case of MAD, due to pathogenic unsafety, the digested sludge was additionally treated by composting before its agricultural application. Therefore, it was transported over 2x40 km to the composting plant and back, so an additional energy consumption of 16 kWh/t of digested sludge for the purposes of composting was included (Li *et al.*, 2020).

Table 2.7 – Inventory data for the sludge line (FU: 1 m³ of produced methane)

Type of data	Item	Referred to FU			Unit
		TAD	MAD	TPAD	
Input data					
energy consumption	AD energy consumption	1.32	1.12	0.79	kWh/m³
	energy consumption for composting	-	0.2	-	kWh/m³
reagents	thickening agent	0.002	0.002	0.001	kg/m³
	anti-foaming agent	0.004	0.008	0.005	kg/m³
	dewatering agent	0.027	0.019	0.014	kg/m³
	chemo dezodoration agent	$1.3 \cdot 10^{-4}$	$1.4 \cdot 10^{-4}$	$8.7 \cdot 10^{-5}$	kg/m³
	bio dezodoration agent	$3.0 \cdot 10^{-4}$	$3.1 \cdot 10^{-4}$	$2.0 \cdot 10^{-4}$	kg/m³
Output data					
wastewater	reject water	0.26	0.26	0.17	m³/m³
wastes	dewatered digested sludge (wet)	11.3	12.3	7.1	kgDM/m³
	dewatered digested sludge (wet)	0.45	0.49	0.28	t*km/m³
avoided products	electricity	3.14	3.14	3.12	kWh/m³
	heat	3.51	3.51	3.49	kWh/m³
emissions	H_2S	0.003	0.003	0.002	kg/m³
	CH_4	0.28	0.29	0.18	kg/m³
	CO_2, biogenic	386	402	253	kg/m³
	CO_2, not biogenic	607	633	398	kg/m³

Among the inputs and outputs, the flows of materials and energy resources, gaseous emissions and solid wastes were considered. When possible, data from a full-scale

facility was used, which was complemented by data gathered in the lab-scale set-up described previously (see the subchapter **"2.3.2 Goal and scope definition"**) (Garfi *et al.*, 2017).

Full-scale data were taken from a WWTP with thermophilic AD operated by Veolia Ceska Republika, a.s. The full-scale operation was used to scale-up and validate the laboratory reactor results. The rest of the information for the MAD and TPAD systems was calculated based on different literature sources and benchmarking data of Veolia, a.s. as described below.

The input parameters included: energy consumption, anti-bulking, anti-foaming and dewatering agent dosage, gaseous emissions from the disposed digested sludges, digested sludge amounts. Calculated parameters were: energy consumption according to the literature (Ferrer *et al.*, 2009), specific biogas production based on benchmarking data of Veolia, a.s., as well as anti-bulking, anti-foaming and dewatering agents' dosages.

The final digested sludge amount for each AD system was estimated in correspondence with the VS destruction observed in the lab-scale digesters, which was consistent with other AD systems studied in the literature (Riau *et al.*, 2010; Courtens *et al.*, 2014; Micolucci *et al.*, 2018).

Since the lab-scale set-up treated WAS and the full-scale plant used sewage sludge with 32.5% WAS, the annual biogas production was recalculated for the thermophilic scenario based on the specific biogas production of the corresponding laboratory reactor.

The heat and electricity production was calculated based on the annual biogas production and technical equipment data (efficiency of combined heat and power unit) given by Veolia, a.s.

Annual gas emissions (CH_4 and N_2O) from the wastewater treatment process and disposed digested sludges were estimated according to the literature (Daelman *et al.*, 2013; Willen *et al.*, 2016).

Background data (chemicals, avoided fertilizers – that are contained in the digested sludge and can be used in agriculture, transportation, wastewater treatment in a municipal wastewater treatment plant, solid wastes, energy provider) were taken from Ecoivent 3.1 database (Garfi *et al.*, 2017). For all electricity requirements, the Czech electricity mix was considered since the full-scale WWTP is located in the Czech Republic (GlobalPetroilPrices.com) (which is composed of: fossil 58.5%, nuclear 35.3%, solar 2.8%, hydro 2.7%, wind – 0.7%).

2.5.3 Impact assessment

The LCA was performed with the software of *SimaPro® 8.2.3*. The potential environmental impacts were calculated by the ReCiPe midpoint method V1.12/Europe Recipe H (Garfi *et al.*, 2017).

Characterisation was conducted for the following environmental impact categories: Climate change, Ozone depletion, Terrestrial acidification, Freshwater eutrophication, Human toxicity, Ionising radiation, Agricultural land occupation, Metal depletion, and Fossil depletion (Lamnatou *et al.*, 2019). The above mentioned nine environmental impact categories were selected and assessed considering their close connection with processes that take place in activated sludge WWTP with AD and that have been used in previous LCA studies (Corominas *et al.*, 2013; Garfi *et al.*, 2017).

Classification and characterisation were performed as the only compulsory steps of impact assessment in terms of standards – ISO 14040 (2006), and ISO 14042 (2006).

2.5.4 Sensitivity analysis

A sensitivity analysis on the digested sludge volume obtained from the TPAD system for both cases WWTP-LCA and SL-LCA was performed in order to take into account the influence that this parameter may have on the environmental impacts associated to digested sludge transport, treatment and reuse.

The sensitivity analysis allowed to evaluate if and how the uncertainty of the assumed value in the inventory table could influence the final results. A variation of ±5% of the digested sludge volume was set for the TPAD scenario only, according to the variability of lab data obtained by the previous studies (Riau *et al.*, 2010; Micolucci *et al.*, 2018; Yu *et al.*, 2020) and shortage of the reported data from full-scale WWTPs, since TPAD is the least spread AD system worldwide among others (Massanet-Nicolau *et al.*, 2015).

This analysis is done through the sensitivity coefficient (S) which indicates the sensitivity of a particular model output to the changes in the variable being considered. The S is calculated according to following equation (Dixon *et al.*, 2003):

$$Sensitivity\ coefficient\ (S) = \left(\frac{/1\$51\$\%@D\%E/1\$51\$F+G}{/1\$51\$2\&,\#F@'\#}\right)/\left(\frac{H'51\$\%@D\%EH'51\$F+G}{H'51\$2\&,\#F@'\#}\right), \qquad (5)$$

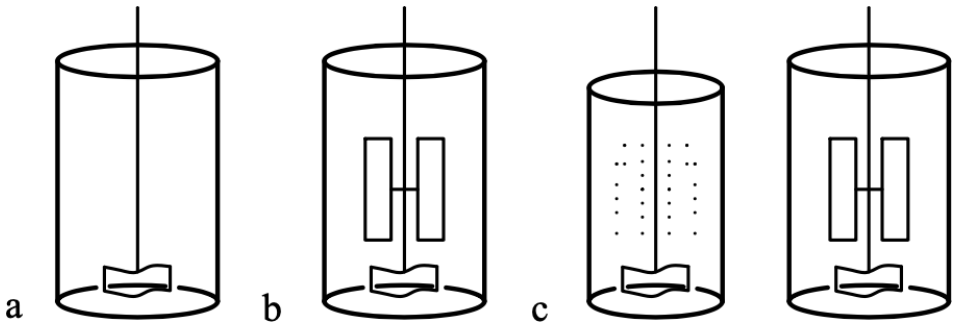
where Input is the value of the input variable (*i.e.* digested sludge amount), and *Output* is the value of indicator according to the correspondent impact category.

Chapter 3 – Results and Discussion

3.1 Mixing efficiency in three types of anaerobic digestion systems: thermophilic, mesophilic and temperature-phased

3.1.1 Basic experimental and modelling results

The installation of three AD systems was tested at the same HRT, within the Phases A-B-D (Table 2.5), and different stirrers (Figure 3.1) and their rotational speed (Table 3.1) based on the experience gained from different literature sources (Lindmark *et al.*, 2014; Kariyama *et al.*, 2018; Zhai *et al.*, 2018; Nguyen *et al.*, 2019).



(a) (b) (c)

Figure 3.1 – Experimental phases A-B-D with different stirring equipment installed: a) mixing system N1 installed in all reactors during the Phase A; b) mixing system N2 installed in all reactors during the Phase B; c) mixing system N2 – inside the TAD and MAD reactors and combined mixing system in TPAD (N1 – inside TPAD1 and N2 – inside TPAD2) during the Phase D

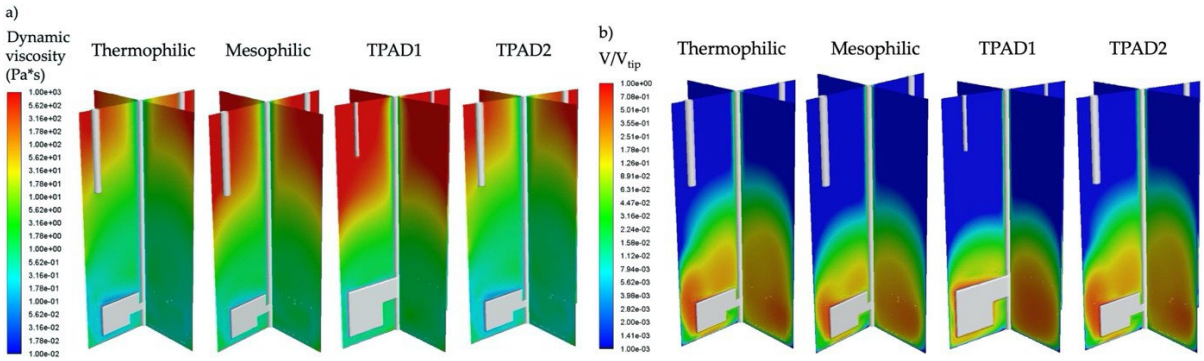
Table 3.1 – Description of the reactors in AD system and their stirring equipment during the Phases A-B-D

Type of reactor	Abbre-viation	Hydraulic retention time (HRT), days	Temperature range during Phase A and Phase B	Temperatu-re range during Phase D	Mixing systems and speed, rpm, at the phases:		
					Phase A	Phase B	Phase D
Single, thermophilic	TAD	13.5	57±1.5°C	57±1.0°C*	N1, 110±1	N2, 50±1	N2, 50±1
Single, mesophilic	MAD	13.5	38±1.5°C	38±1.0°C*	N1, 110±1	N2, 50±1	N2, 50±1
Double-stage, thermophilic, (the first stage)	TPAD1	2.0	57±1.5°C	57±1.0°C*	N1, 95±1	N2, 30±1	N1, 30±1
Double-stage, mesophilic (the second stage)	TPAD2	11.5	38±1.5°C	38±1.0°C*	N1, 110±1	N2, 50±1	N2, 50±1

Note: *All digesters at the Phase D were covered with the insulation (Table 2.4).
**All digesters at all Phases were continuously mixed at the fixed speed.

Within the first working weeks of the Phase A, it was observed that the biggest part of the reactor volume was not properly mixed – according to the visual assessment, up to 2/3 of the working reactor volumes was not moving. It is important to mention that the rotating speed was adjusted and fixed at 110 rpm for TAD, MAD and TPAD2 and 95 rpm for TPAD1 in order to provide favourable mixing conditions for as big portion of working reactor volume as possible (Zhai *et al.*, 2018).

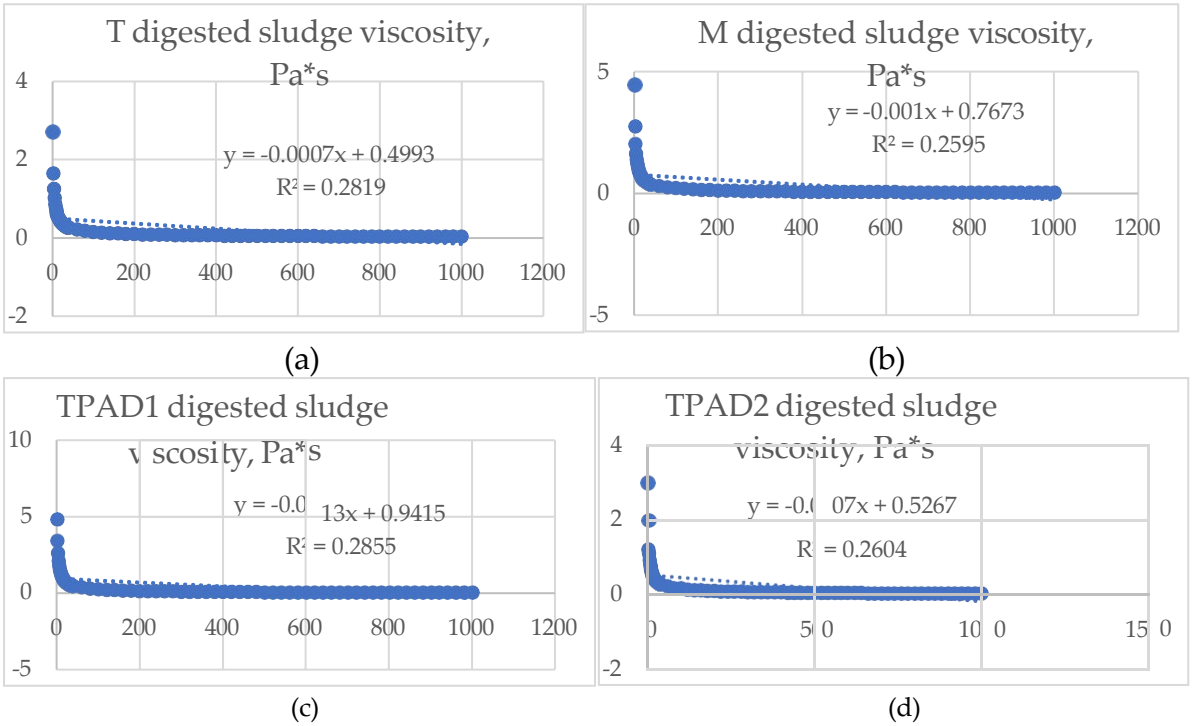
This visual observation is in close agreement with the results of CFD simulation as shown in the Figure 3.2, where are presented results of the sludge viscosity for two vessels sizes operated also at 110 rpm (TAD, MAD, TPAD2) and 95 rpm (TPAD1).



(a) (b)

Figure 3.2 (a-b) – Mixing efficiency models at the Phase A of TAD, MAD, TPAD1, TPAD2 sludges: a – as a function of dynamic viscosity; b – the velocity profiles

Those results were based on the sludge viscosity data experimentally obtained and presented in the series of the graphs (Figure 3.3):



(a) (b)

(c) (d)

Figure 3.3 (a-d) – Digested sludge dynamic viscosity measured using rotational rheometer RC20 (working parameters are rotation ramp measuring block, controlled shear rate, linear shear rate – 0 -> 40 1/s, testing time – 300 s, amount of point measurements – 30 points, without temperature control)

The sludge viscosity showed strong dependency of the viscosity with increasing shear rate, with clear shear thinning effect. This dependency was used in all our simulation to approximate viscosity variation as a function of mixing conditions used in the simulations.

As it can be seen, there was large zone at the top of the vessel (Figure 3.2) where the viscosity reached extremely high values, reflecting observed stagnant fluid. The reason for such string effect was related to the shear thinning effect of the sludge. The "dead" zone location was similar for all digesters, but its area was different for every reactor type in a descent order: TPAD1, with the lowest HRT and highest viscosity, then at the MAD reactor and, finally, it was quite similar at TAD and TPAD2 digesters.

It is also can be seen how the digested sludge viscosity influenced the velocity profile of the reactors (Figure 3.2). The shapes of intensively mixed zone and, consequently, lower viscosity were similar. However, these two zones did not completely match each other: the velocity range fades more clearly, so that the well-mixed zone was smaller, than the zone of lower sludge viscosity.

Based on all above mentioned it can be clearly stated that all reactors were not mixed well enough.

Despite not sufficient mixing in the MAD and TAD during the Phase A, the specific biogas production was satisfactory, with respect to the substrate used (Table 3.2).

Table 3.2 – Specific methane production within the whole operational period

Phase	Stirring system type	TAD	MAD	TPAD1	TPAD2	TPAD
Phase A	N1*	0.10± 0.002	0.09± 0.001	0.03± 0.001	0.18± 0.002	0.22
Phase B	N2*	0.07± 0.002	0.07± 0.003	0.06± 0.002	0.08± 0.001	0.15
Phase C	N2*	0.17± 0.001	0.16± 0.001	0.05± 0.001	0.19± 0.001	0.23
Phase D	N1 + N2*	0.12± 0.001	0.14± 0.003	0.04± 0.002	0.16± 0.001	0.19

Note: *Mixing system N1 was inside TPAD1; N2 was inside TAD, MAD, TPAD2 during the Phase D.

At the same time, TPAD system performed better on about 50% at the Phase A, around 55% at the Phase B and 30% at the Phase D in terms of the specific methane production in comparison to TAD and MAD digesters, respectively.

At the Phase B, the specific methane production dropped on around 30% for TAD, 20% for MAD and 30% for TPAD. In addition, the difference in specific methane production between TPAD1 and TPAD2 went down from 80% to 25% at the Phase B.

It can be noticed that TPAD system behaviour changed in a very interesting way during the Phase B. The amount of methane raised up almost twice in the fermenter (TPAD1) and dropped mainly in the second stage of the system – on 60% from the previous average value obtained during Phase A (Table 3.2). That meant that the additional vertical paddle provoked a disbalance of methane production system in TPAD. It rendered the higher biogas and, respectively, methane production in TPAD1 – instead of TPAD2 – the stage, which was supposed for intensive methanogenic activity. Thus, the change in the mixing system in both TPAD reactors was paradoxically negative for TPAD system as a whole concept, as the main advantage of TPAD system is the location of methanogenic process in the second stage TPAD, separately from the acidification steps happening prior to it in TPAD1. It can be stated that the main principle of double-stage AD system during the Phase B was completely supressed.

At the Phase D, TAD performance was improved on approximately 20% in relation to the Phase A and 40% in relation to the Phase B; MAD performance was improved on 35% in relation to the Phase A and 50% in relation to the Phase B. The disbalance of the specific methane production between two stages of TPAD went down at the Phase D, as it was observed at the Phase A and became 75%. Nevertheless, the overall specific methane production during the Phase A was the best – on almost 15% better than at the Phase D. At the Phase B, it was registered on around 20% lower than at the Phase D.

The Phase B methane drop can be explained by higher rotational speed applied for the better impeller construction (Lemmer et al., 2013) than at the Phase A.

The obtained data of the specific methane production went along with the volatile solids' destruction – Table 3.3.

Table 3.3 – The organic matter destruction during the Phases A-D

Phase	Parameter	TAD	MAD	TPAD1	TPAD2	Substrate
Phase A, HRT = 13.5 days	VSS (g/L)	20.2±1.5	23.4±1.5	26.1±2.7	19.4±1.0	42.9±1.1
	VSS_{rem} (%)	52.9	45.4	39.1	54.8	-
Phase B, HRT = 13.5 days	VSS (g/L)	21.6±1.2	23.7±0.7	27.6±0.6	20.8±0.8	39.9±2.1
	VSS_{rem} (%)	45.9	40.6	30.8	48.0	-

Phase C, HRT = 19.0 days	VSS (g/L)	21.0±1.8	25.2±2.6	28.0±3.0	22.2±1.7	37.5±3.4
	VSS_{rem} (%)	43.9	32.7	25.3	40.8	-
Phase D, HRT = 13.5 days	VSS (g/L)	21.1±0.6	23.4±1.0	28.6±1.2	20.7±0.6	41.5±3.5
	VSS_{rem} (%)	49.1	43.6	31.1	50.2	-

The highest organic matter removal rate is registered at the Phase A for TAD and TPAD systems. MAD system represented relatively stable degradation rate at the Phases A-B-D.

After changing the mixing system from N1 to N2, TPAD1 showed the better results in organic matter degradation rate, which also resulted in higher methane production in the fermenter (Table 3.2). Applying the insulation improved the performance of the second stage TPAD2, however, the best total performance was monitored during the Phase A.

In addition, the visual medium separation along with temperature stratification within the digesters of MAD and TAD appeared. According to Nguyen *et al.* (2019), these phenomena may result in potential acceleration of the foaming due to the bad substrate distribution along the reactor volume and surface-active substances accumulation on the top of the reactor medium. Secondly, there was a distinctive difference in microbial community structure at the top and bottom of reactor.

The rare foaming events happened to MAD reactor and TPAD1 during all Phases, the most severe took place during the Phase A. At those events, foam captured up to around 15% for TPAD1 and MAD of reactor head space at maximum. TPAD2 performance demonstrated the least foaming potential. Thus, the operationally significant foaming was not detected at the Phases A-B-D that would lead to complete malfunction of any digester.

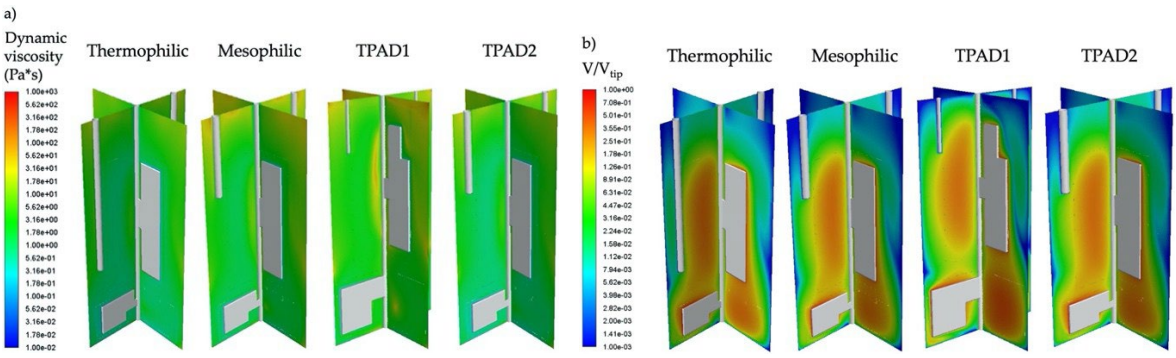
Temperature stratification was registered due to two temperature sensors installed in the reactor (Appendix A, Figures A.1.1 and A.1.2). The first sensor was used as an indicator for the thermostat, and it was installed closely to the reactor bottom (around 6.0 cm – for TPAD1 and 11.0 cm – for the other reactors). The second probe measuring the medium temperature was a pH-probe with incorporated thermometer. This sensor was placed on the depth of 16.5 cm from the inner surface of every reactor lid. Meanwhile the medium separation into liquid and sludge fractions was slight and did not reach even 1.0 cm from the medium interface to the medium surface, the temperature stratification grew up to 10 ºC for T reactor and 7 ºC – for the fermenter (TPAD1) of TPAD system which was run also under T conditions. For both mesophilic reactors, it was detected not more than 4 ºC of temperature difference between those two measuring points. After the vertical paddle was added, there were no temperature stratification detected at a range higher than the temperature fluctuations

due to the heating system arrangement at each Phase of the whole period of experimental work (±1.5 for the Phase A, B, C and ±1.0 – for the Phase D).

During the Phase A, the biogas and methane production was rather stable. However, it seemed to have certain space for improvement due to the inefficient mixing.

Therefore, according to the CFD simulations presented in the Figure 3.2 and due to strong shear thinning effect of the media it was decided to modify the AD systems with an additional vertical paddle (Appendix A, Figures A.1.1 and A.1.2) and change the stirring regime – to reduce the rotational speed up to 50% (Table 3.2) – as it was also tried out by. (Zhai *et al.*, 2018; Kariyama *et al.*, 2018; Ma *et al.*, 2019).

The stirring equipment modification in its shape and geometry was made to introduce a possibility to mix the medium at lower rotational speed to prevent biochemical processes intervention inside the reactors and, at the same time, better homogenization within the whole working volume of the reactors. Achieving those two aims, one more paddle of vertical paddle was added to each reactor (Figure 3.1 and Appendix A, Figures A.1.1. and A.1.2). Simulation results of the Phase B are presented in the Figure 3.4, where is reported how the viscosity changes and velocity profile distributes inside all four studied vessels (TAD, MAD, TPAD1, TPAD2) operated at different stirring speed.



(a) (b)

Figure 3.4 (a-b) – Mixing efficiency models at the Phase B of TAD, MAD, TPAD1, TPAD2 sludges: a – as a function of dynamic viscosity; b – the velocity profiles

In fact, by adding additional paddle at the top of the vessel there was significant improvement of the mixing associated with the decrease of the media viscosity. There was almost no very high viscosity zone (Figure 3.2). Interesting is the fact, that such improvement was achieved while reducing the impeller stirring speed.

Looking at the velocity range in the digesters (Figure 3.2), it can be said that the main body of the reactor was well mixed, and there were no vast "dead" zones anymore. Due to the better mixing process, it was expected to observe a better reactor performance. Indeed, the medium separation and temperature stratification

disappeared. However, according to the monitored parameters – methane production (Table 3.2) and organic matter destruction (Table 3.3) for all Phases – the specific methane production dropped for all AD systems after changing the stirring system from N1 to N2 and the regime of mixing (Figures 3.2-3.6).

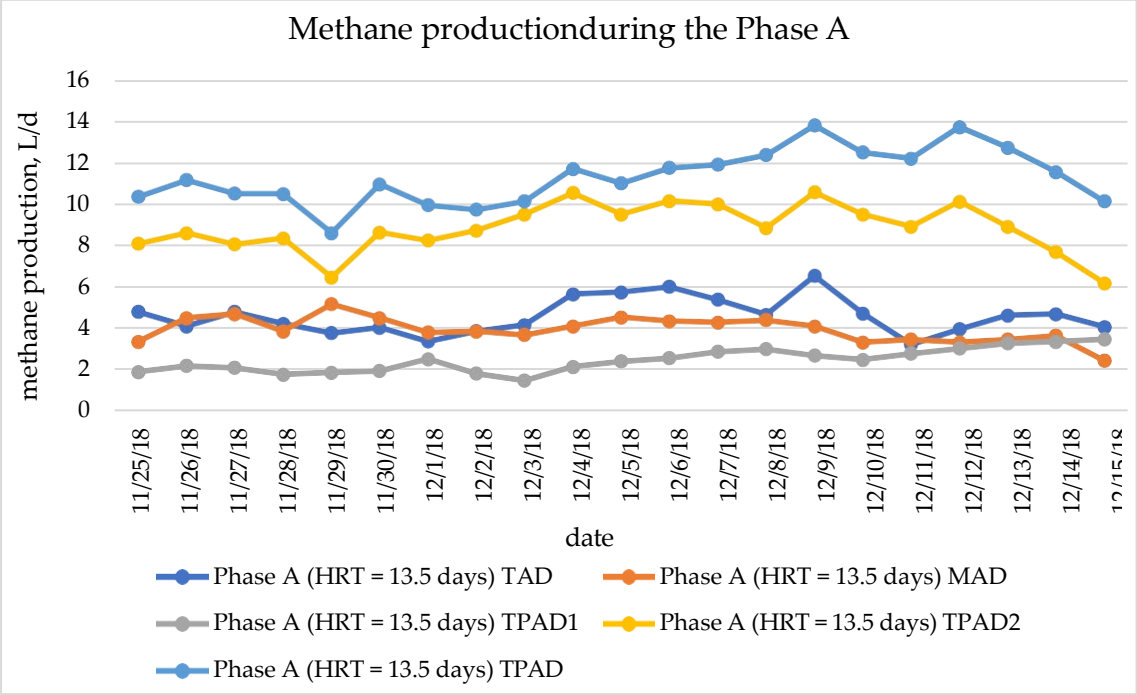


Figure 3.4 – Methane production at the Phase A, 25.11.18-15.12.18

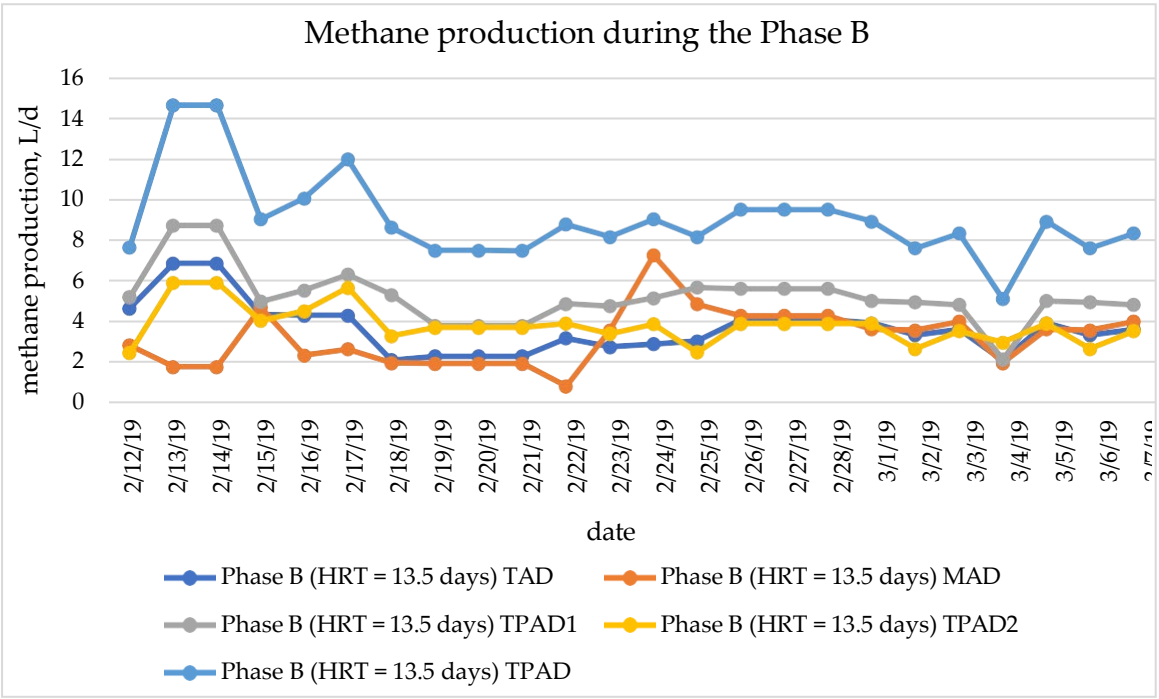


Figure 3.5 – Methane production at the Phase B, 12.02.19-07.03.19

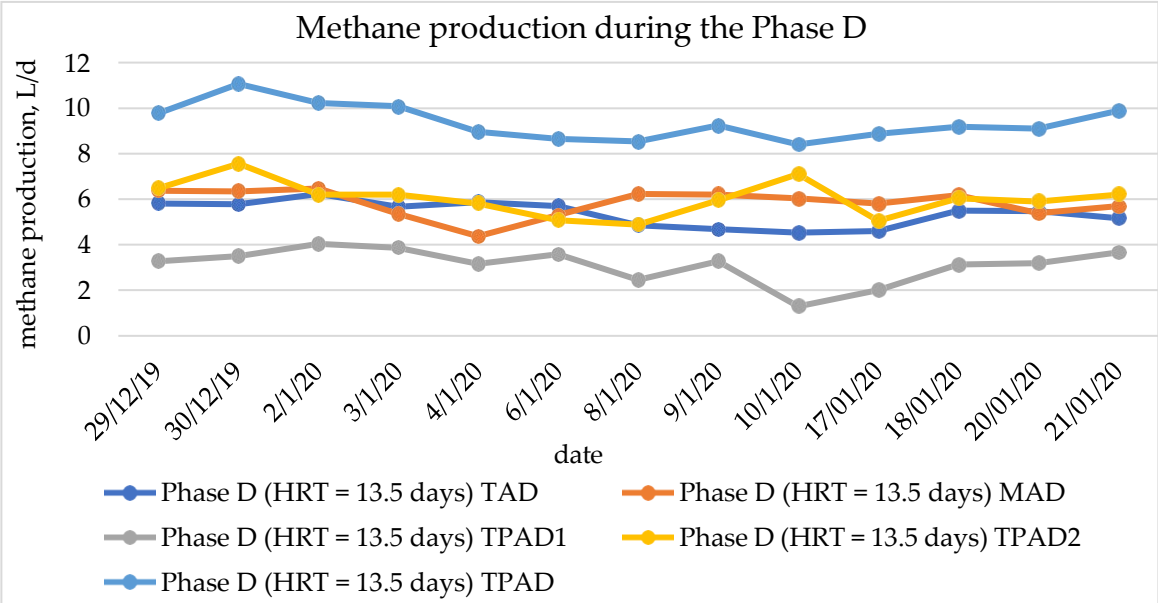


Figure 3.6 – Methane production at the Phase D, 12.02.19-07.03.19

All above mentioned underlines the importance of not only a type and shape of a mixing device, but also the stirring regime, namely its intensity, with regard to the content and quality of the medium (its viscosity) and a function of a mixing process.

The Table 3.4 shows the comparative performance of the AD systems in terms of the specific methane production between the initial Phase (Phase A) as a milestone and two others (Phases B and D).

Table 3.4 – Comparative performance of the AD systems between the Phase A and Phases B-D in terms of specific methane production

Phase	TAD (%)	MAD (%)	TPAD1 (%)	TPAD2 (%)	TPAD (%)
Phase A	100	100	100	100	100
Phase B	-31.4	-13.7	+48.1	-60.6	-27.1
Phase D*, **	+14.2	+35.1	+22.6	-30.2	-17.2

Note: *All digesters at the Phase D were covered with the insulation (Table 2.4).
**Mixing system N1 was inside TPAD1; N2 was inside TAD, MAD, TPAD2 during the Phase D.

All reactors were influenced significantly and, mainly, negatively in terms of methane production by changing the mixing equipment, according to the Table 3.4. Nevertheless, the data presented in Table 3.4 underline sensitivity of digester TAD. One can observe the decrease in methane production by over 30% at the Phase B. The most stable performance is presented by MAD reactor with the least drop of methane production.

It was also noticed that TPAD system behaviour changed in a very interesting way during the Phase B (Figure 3.7).

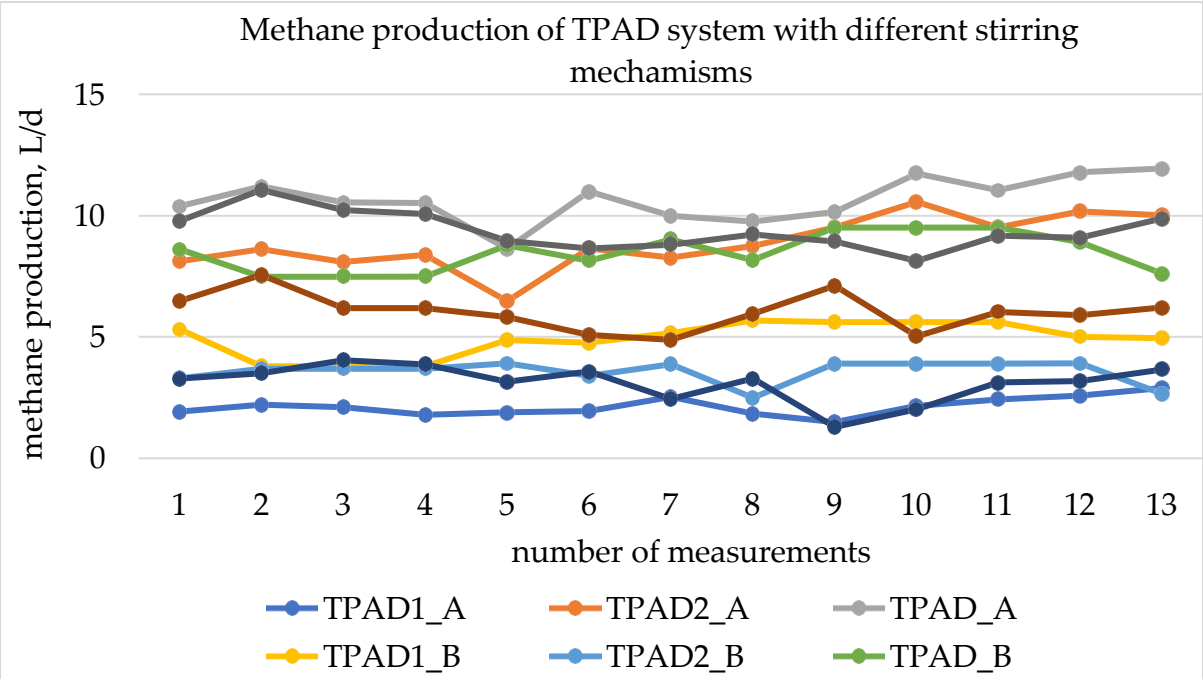


Figure 3.7 – Methane production of TPAD system with different stirring mechanisms
*Note: A – with installed N1 stirring mechanism; B – with installed N2 stirring mechanism; D – TPAD1 with N1 stirrer (the second paddle was removed) and TPAD2 – with N2

The amount of methane raised up almost twice in the fermenter TPAD1 and dropped mainly in the second stage of the system – on 60% from the previous average value obtained during Phase A (Table 3.4). That meant that the additional vertical paddle provoked a disbalance of methane production system in TPAD. It rendered the higher biogas and, respectively, methane production in TPAD1 – instead of TPAD2 – the stage, which was supposed for intensive methanogenic activity. Thus, the change in the mixing system in both TPAD reactors was paradoxically negative for TPAD system as the whole concept, because the main advantage of TPAD system is the location of methanogenic process in the second stage TPAD, separately from the acidification steps happening prior to it in TPAD1. It can be claimed that the main advantage of double-stage AD system during the Phase B was significantly supressed. All above mentioned negative effect of the change from mixing system N1 to N2 and rotational speed decrease might occur due to the factor based on the hypotheses:

a) the stirring velocity could either be not lowered enough, or enhanced by elaborated daily mixing regime (Kariyama *et al.*, 2018; Zhai *et al.*, 2018);
b) regarding TPAD, there might be no need to change the mixing system in both TPAD digesters.

Hence, by adding a new vertical paddle to each reactor, it turned out to be possible to avoid temperature stratification and medium separation. However, it did not affect the overall temperature fluctuations, which was ±1.5 °C (Table 2.1), which was too high and could lead to methanogenesis inhibition, especially the thermophilic

digesters (Pasalari *et al.*, 2020). With regard to the Phases A and B, there was left a question of regime temperature maintenance of any AD system. In addition, TPAD system as the most complicated according to its configuration, showed the worst results (Table 3.4) and demanded further investigation.

Afterwards, it was decided to run one more phase – the Phase D in order to reach two objectives:

1. to improve the performance of all AD reactors by better temperature regulation – adding the insulation;
2. to study TPAD system at new mixing conditions.

The three-layer insulation (Table 3.1) could explain the better performance of all AD systems in terms of methane production comparison to it during the Phase B, when the mixing systems and regimes were similar, excluding TPAD1 (Table 3.4). However, one-month run prior to the Phase A and one-year of permanent reactor performance prior to the Phase D should be also considered.

It can be stated that the Phase D provides us with the best possible results for T and M conditions in terms of methane production (Table 3.2): with the stirring equipment N2 and proper insulation.

This means that having the same mixing system, a proper insulation cover decreases temperature fluctuations (Table 3.1) and promotes not only process stability, but also efficiency of methanogenesis. Meanwhile, at the Phase D, the organic matter degradation extent is not higher than at the Phase A (Table 3.3).

By removing the vertical paddle from TPAD1 and adding proper insulation at the Phase D (Figure 3.6) the biogas balance was partly improved between two stages of TPAD. Which was also proved by CDF simulation models (Figure 3.8).

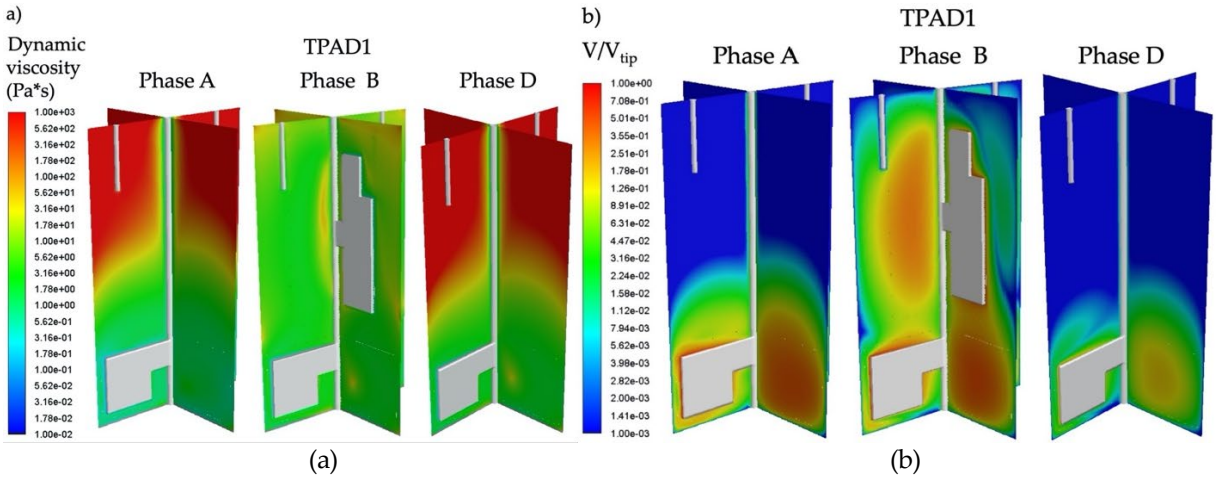


Figure 3.8 – Mixing efficiency models of TPAD1 at the Phases A-B-D: a – as a function of dynamic viscosity; b – the velocity profiles

However, the methanogenic activity in TPAD1 remained too high which did not allow to improve the total TPAD performance significantly and reach, at least, the initial methane production efficiency registered at the Phase A (Tables 3.2 and 3.3)

That might happen because of two reasons:

1) the HRT of TPAD1 was long enough to keep methanogens in the digester (Fernández-Rodríguez *et al.*, 2016);
2) the biofilm containing methanogens was formed on the inner surfaces of TPAD1 due to the long-term operation.

The methane production efficiency in TPAD system was the highest during the Phase A comparatively to the Phases B and D. During the Phase A, TPAD system performance was also the best among other AD systems presented in our studies (Table 3.2).

All above mentioned mean that changing the mixing system configuration and applying correct temperature maintenance can lead to the following conclusions:

a) avoiding temperature stratification and medium separation;
b) showing that temperature stratification and medium separation are not the straight signs of bad reactor performance. These parameters are the indicators of the fact that the mixing conditions are not optimal;
c) appropriate insulation of digesters is one of the basic conditions of temperature and process stability and, consequently, the degradation efficiency of AD and influences significantly more than the upgrade of the mixing equipment;
d) all AD systems are very responsive to the change of mixing equipment;

d.1) the single-stage TAD reactor is more vulnerable to mixing conditions than the single-stage MAD one;

d.2) both stages of TPAD system are highly interconnected and comparable to the single-stage AD systems in terms of stirring sensitivity.

In addition, according to the literature, not only type of mixing mechanism and rotational speed, but also mixing regime (stirring duration, interval and intensity) might be a smart solution (Lindmark *et al.*, 2014).
Focusing on TPAD, it was important not only to highlight the best efficiency during Phase A, but also to study the ratio of methane production in between stages of TPAD system separately.

After changing from N1 to N2, the methane production disbalance that appeared during the Phase B was not completely removed at the Phase D. That could be beneficial, if the methane production in TPAD2 within the Phase B would stay at the same level as it was monitored at the Phase A. Hence, it was decided to sample

biomass from both reactors of TPAD system after the experimental period of the Phase B and before the Phase D to clarify two objectives:

1) to determine why mixing system N2 caused significantly higher methane production without changing relatively short HRT, equal to two days during all Phases;
2) to define which consortia are responsible for methane production in TPAD1 (at two sampling points – suspended medium and attached to the reactor wall) and TPAD2 (only suspended matter).

3.1.2 Microbiological analysis

3.1.2.1 Occurrence assessment by FISH and core community in digesters

According to FISH, bacteria were found in all three samples D1-D3 (D1 – suspended biomass in TPAD1; D2 – biofilm in TPAD1; D3 – suspended biomass in TPAD2), but not in significant amount in comparison to archaea. The abundance was assessed based on the general screening, not quantification. The used scale range from one to three pluses, where the more plusses the more organisms presenting positive fluorescent signal were observed (Appendix B, Tables B.1.1. and B.1.2.). The limitation of preliminary FISH analyses performed in our study is the unspecific binding of probe ARCH915. This was proven by Gagliano *et al.* (2015) for *Coprothermobacter* spp. In all samples investigated with 16S that was 1st/2nd most abundant species in sequenced samples (Appendix B, Figure B.1.3). The significant abundance of methanogens was confirmed in 16S analyses. According to our results and difficulties in obtaining representative samples for biomass thermophilic reactor, it was decided to analyse the relative abundance and microbial composition with 16S amplicon sequencing.

On the other hand, FISH was useful to localize the highest abundance of the methanogens in the TPAD1 system. It was not detected at any level in suspended sludge, therefore, other areas of interest were the biofilm from the central part of the reactor and the wall of the reactor. The archaea were so abundant there that we decided to include this sample in further analyses.

The sequencing results were very satisfying and allowed to identify the majority of digesters' biocenosis. It was sequenced three samples from the AD digesters with 152610 total number of reads and distinguished 869 unique OTUs. 100% of OTUs were assigned to the kingdom level, more than 92% to phylum (97.58%) and class (92.75%), over 80% to order (86.31%) and family (82.8%) and over 72% to genus (Table 3.5).

Table 3.5 – Information about sample characteristics*

Sample Name	Extraction Conc. [ng/uL]	Library Conc. [ng/uL]	Reads	Observed OTUs	Shannon index
D1	15.5	2.5	50500	477	4.2
D2	16	4.6	48722	429	4.3
D3	24.5	3.4	53289	447	4.3

Note: *Reads is the number of reads after sequencing, QC and bioinformatic processing. Observed OTUs is the number of observed OTUs in each sample, in order to compare the samples it is calculated based on 10,000 reads pr. Sample. Shannon index was calculated based on 10,000 reads pr. Sample to facilitate comparison among samples.

The mean abundance (in percentage of pair of reads) in the digested sludge is shown in the Supplementary material (Appendix B, Figures B.1.1-B.1.3).

Forty most abundant genera constituted 3/4 of the microbial community (74.1%, 76.2%, 77.3% in D1, D2, D3, respectively). The groups' abundance varied from 0 to 22.3%. Study on activated sludge samples done by (Miłobedzka and Muszyński, 2017) using similar sequencing method revealed higher diversity in bacterial community in activated sludge and even though only half of the biocenosis was characterized it was still useful for comparison among the treatment systems. While in study in digesters ten most abundant groups belonged to only six phyla (Appendix B, Figure B.1.1).

Among those phyla, Bacteroidetes and Firmicutes are the most abundant in full scale anaerobic digesters in Denmark (Kirkegaard *et al.*, 2017). Euryarchaeota (*Methanosarcina*) was dominant archaeal phylum both in our study and in Danish digesters (Volkmann *et al.*, 2007; Kirkegaard *et al.*, 2017). On the other hand, in the study by Li *et al.* (2018) focusing on the lab scale AD Firmicutes were the dominating bacteria. However, in our study, the other major group were Proteobacteria, not the Bacteroidetes, which found by Li *et al.* (2018) in the mesophilic continuously stirred tank reactor. There were reported abundance above 1% in tested digesters for 11 phyla. Five of them: Firmicutes (>36%), Proteobacteria (>11%), Actinobacteria (>4.7%), Bacteroidetes (>4%), Chloroflexi (>1%), were also numerous in digesters investigated in this study. Similar to Liu *et al.*, (2019), we observed domination of *Clostridia*, reported for 30.4–44.3% (Appendix B, Figure B.1.2.), conferring similar core community in all AD systems.

All samples sequenced for this study had high Shannon index and number of identified OTUs (Table 3.5). High Shannon indexes for the separated stages in digestion, systems TPAD1 and TPAD2 seem to confirm that such segregation enriches and diverse microorganisms in digesters (Shabbir *et al.*, 2017). This biodiversity stabilizing the system and promoting high processing performance has been connected by Liu *et al.* (2019) to explicit result of hydrolysis, acidogenesis and methanogenesis of three stage in three-stage anaerobic digestion. This also contributes to the higher availability of the substrates (VFAs and ammonia) for the anaerobic

bacterial growth (see the Tables 3.7 and 3.10 in the subchapters *"***3.1.4 VFA content***"* and *"***3.1.6 Free ammonia and ammonia nitrogen presence***"*).

1.2.2 TPAD1 and TPAD2 suspended biomass comparison

The biggest differences in abundance among forty analysed genera were noted for *Coprothermobacter* (selectively growing in digesters) and *Tepidimicrobium*, *Defluviitoga*, *Methanosarcina* as well as *Tepidiphilus* (growing in thermophilic anaerobic digesters, found only in D3).

Coprothermobacter and *Tepidimicrobium* were the most abundant bacteria in TPAD1, almost twice more abundant than in TPAD2. *Coprothermobacter* are associated with methanogenic microbial communities in thermophilic anaerobic digesters. In our study, these bacteria were significantly more abundant than in Danish full scale thermophilic digesters (Nierychlo *et al.*, 2020), similarly to *Tepidimicrobium*. It stays in agreement with finding that growth of *Coprothermobacter* in anaerobic digesters is determined by substrate type as well as thermal pre-treatment (Gagliano et al., 2015; Tandishabo et al., 2012)(Tandishabo *et al.*, 2012; Gagliano *et al.*, 2015).

Defluviitoga were more abundant than in the full-scale Danish mesophilic and average thermophilic digesters. *Methanosarcina* in our study constituted higher fraction than in average Danish mespohilic digesters, but smaller than in thermophilic ones.

Similar abundance of *Diaphorobacter*, *Simplicispira* and *Tetrasphaera* was observed in both systems, which can be mirroring abundance of those species in the primary sludge. *Thermomonas*, K2−30−37 (Chlorobi) and f_RFN82_OTU_14 (*Tenericutes*) were more abundant in TPAD1, it can be somehow connected to the feeding regime (Svensson *et al.*, 2018).

Methanogens and acetogens in TPAD1 and TPAD2 were classified according to MiDAS 3 ecosystem-specific reference database by Nierychlo *et al.* (2020). *Anaerobaculum* was the only acetogen in the study. Those are moderately thermophilic anaerobes and chemoorganotrophs. They produce acetate, hydrogen and CO_2 in fermentation of organic acids, proteins and some carbohydrates (Menes & Muxí, 2002; Maune & Tanner, 2012). *Methanosarcina* are strictly anaerobic methanogenic Archaea. They are capable of using substrates on acetoclastic, hydrogenotrophic and methylotrophic pathways, they use acetate, H_2/CO_2, methanol for growth and methylamines as source of energy (Sowers *et al.*, 1984; Elberson & Sowers, 1997; Lyimo *et al.*, 2000; Von Klein *et al.*, 2002; Singh *et al.*, 2005; Shimizu *et al.*, 2011; Di Maria *et al.*, 2017). *Methanothermobacter* are strictly anaerobic hydrogenotrophic archaeal methanogens converting H_2 and CO_2 to methane (Wasserfallen & Macario, 2000; Cheng *et al.*, 2011). *Methanothermobacter* and *Methanosarcina* were previously connected in full scale plants to the thermophilic systems (Kirkegaard *et al.*, 2017). The later one endure wide range of operating temperature in digesters, it can be also

connected to shorter residence times (De Vrieze *et al.*, 2012). Both studies by Jang *et al.* (2015) and Liu *et al.* (2019) recorded connection between feed rich in dissolved organic matter and high abundance of hydrogen-utilizing methanogens.

Methanosarcina, *Methanothermobacter* and *Anaerobaculum* were more abundant in TPAD2 (12.1, 1.1 and 2.5, respectively) than in TPAD1 (in biofilm 2.5, 0.3 and 0.3, in suspension 0.2, 0.2 and 0.1, respectively). This proves the fact that hydrogen content at TPAD2 was continuously and sufficiently lower than at TPAD1 (see the Table 3.9 in the subchapter "**3.1.5 Hydrogen content**"). We can also observe enrichment on those organisms in the biofilm in TPAD1 which turned out to be around 10% of total working reactor volume (1.45 L).

1.2.3 Bacterial distribution in TPAD1

D1 is representative for suspended biomass and D2 for biofilm in TPAD1. The biggest differences in abundance were observed for *Methanosarcina*, *Syntrophomonas*, *Petrimonas* and some Bacteroidetes (vadinBC27 wastewater−sludge group) constituting 2.5-16.6% in biofilm in comparison to only 0.1-0.2% in bacterial suspension.

Bacteroidia, were less abundant than in Liu *et al.* (2019), but still the second most numerous group in biofilm in TPAD1. Also, the higher abundance of *Cloacimonetes*, *Petrimonas* and *Proteiniphilum* in the biofilm could have enhanced the digestion process. As those bacteria are capable of fermentation using short chain fatty acid, sugars and proteins, one can speculate that those bacteria improved the processing of complex molecules to simpler compounds in TPAD1 and developed its performance. Also boost the methanogenesis own to high abundance of *Methanosarcina*.

The biofilm was formed on the inner wall of TPAD1 and on the surface of the stirring equipment within more than three-month operation. Up to 2.0 cm biofilm over 0.011 m^2 of the inner wall and paddle surface in TPAD1 (Appendix B, Tables B.1.1. and B.1.2.) protected the methanogenic consortia that could survive within significantly longer period of time than HRT of two days in the same volume of fermenter under around 1.20 gCOD/(gVSS.d) of organic loading rate with a total load of TPAD of 1.18 gCOD/(gVSS.d) (Table 2.5). This little, but very distinctive difference between the microbial community inside one reactor volume explains why there was such intensive methane production in TPAD1 in all phases of operation and especially after increase of paddle surface. This shows that certain type and design of the stirring equipment installed inside a lab scale reactor affects AD process significantly in the context of microbial consortia natural selection (Meister *et al.*, 2018).

3.1.3 pH measurement

The pH profile at the experimental Phases A-B-D reflects the progress of organic matter degradation (Table 3.3) at each phase and over the whole period of monitoring (Table 3.6).

Table 3.6 – The registered average pH values*

Phase	TAD (-)	MAD (-)	TPAD1 (-)	TPAD2 (-)
Phase A	7.40±0.06	7.45±0.02	6.89±0.13	7.63±0.02
Phase B	7.76±0.20	7.31±0.09	7.26±0.26	7.66±0.06
Phase C	7.81±0.04	7.44±0.03	7.19±0.13	7.84±0.04
Phase D**, ***	7.95±0.06	7.70±0.05	7.18±0.14	7.98±0.04

*Note: Averaged values were obtained from the online logged-in data with the frequency of once per hour, during the whole period of experiments.
**All digesters at the Phase D were covered with the insulation (Table 2.4).
***Mixing system N1 was inside TPAD1; N2 was inside TAD, MAD, TPAD2 during the Phase D.

It can be seen that at the beginning of the experimental period – at Phase A – the pH value range was not very different due to the insufficient mixing. The highest standard deviation period referred to the Phase B, as the most unstable. At the same time, with longer operation period and better mixing efficiency, the differentiation of averaged pH values started to grow. After one year of reactor running, the average pH values reached the most stable positions.

3.1.4 VFA content

Volatile Fatty Acids (VFA) are of specific concern, as they are considered to be one of the valuable products that can be recovered from the digesters and used for biofuels or bioplastics production (Wang *et al.*, 2014; Bermúdez-Penabad *et al.*, 2017; Li *et al.*, 2018). The data of VFA production is depicted for each Phase of the experiments (Table 3.7).

Table 3.7 – VFAs' concentrations in the digested sludges within the Phases A-D

Phases	TAD (mg/L)	MAD (mg/L)	TPAD1 (mg/L)	TPAD2 (mg/L)
Phase A	2738±320.5	308±23.7	5219±447.3	438.6±125.2
Phase B	1356±209.5	467±55.3	9187±1888.0	492.5±70.4
Phase C	2053±262.6	519±184.5	7452±1777.7	522±145.4
Phase D	2821±195.0	329±43.2	7810±534.0	366.8±17.9

After the Phase A, the VFA concentration reached certain peaks for the three of four reactors: +34% for MAD, +43% TPAD1 and +11% for TPAD2. Unlike others, the TAD reactor performed less efficient on over 50% comparing to the Phase A. In the case of TAD, the methane production also dropped (Table 3.2) because of accelerated process of hydrolysis. TAD is a single-stage reactor, thus, the increased VFA concentration

hampered methanogenesis, happening in the same reactor, what did not take place in a double-stage TPAD.
At the Phase B, the methane production was inhibited at all reactors, besides TPAD1 (Table 3.2) due to the biofilm formed on the walls and mixing equipment (see the subchapter "**3.1.2 Microbiological analysis**"), the surface of which significantly bigger comparatively to those of TAD, MAD and TPAD2. Thus, HRT in the formed biofilm was much longer than two days.

Apparently, for other cases, apart from TPAD1, the change of the mixing equipment led to the disturbance of the microbial community. As it is known, the thermophilic methanogenic microorganisms are the most sensitive (Cai *et al.*, 2021). By putting an additional vertical paddle, the proces of hydrolysis was accelerated. At the same time, the methanogenic biomas was disturbed. Thus, the biomass could not completely adapt to the new stirring circumstances over the whole period of the experiments – three months (Tables 3.2 and 3.3).

It can be quite vividly seen that at the Phase C, VFA production was increased at all AD systems, besides TPAD1. Apparently, at longer HRT (the Phase C was run at 19.0 days of HRT), the accumulation of FVA was observed.

At the Phase D, the mixing conditions for TAD and MAD were left the same as it was at the Phases B and C. The VFA production went back, almost to the state of the Phase A. It is, supposedly, due to a long term of biomass accommodation to the mixing system N2 after N1. Which indicates the sensitivity of both TAD and MAD reactors to the mixing conditions. Additionally, the sensitivity of the thermophilic methanogenic consortia is very high, as the change of the mixing equipment provoked serious disruption of the AD system at the Phase B (Table 3.2 and 3.7).

Besides the VFA concentration, the data of VFA type distribution are of the interest (Table 3.8).

Table 3.8 – VFA content in AD systems

Phases	TAD (%)			MAD (%)			TPAD1 (%)			TPAD2 (%)		
	C2*	C3*	C4+n*	C2*	C3*	C4+n*	C2*	C3*	C4+n*	C2*	C3*	C4+n*
Phase A	20.8	53.7	25.5	63.2	18.7	18.1	28.9	34.0	37.2	60.3	16.9	22.8
Phase B	62.3	18.5	19.3	60.9	11.5	27.6	28.9	23.9	47.2	65.1	8.6	26.3
Phase C	40.5	19.9	39.6	57.9	16.3	25.8	29.7	23.9	46.4	63.9	12.4	23.7
Phase D	11.6	51.5	37.0	70.6	21.2	8.2	31.9	22.2	45.9	72.0	10.9	17.1

*Note: C2 – acetate; C3 – propionate; C4+1 – the sum of other VFAs such as butyrate, isobutyrate, valerate, isovalerate, hexanoate in case of their availability

For the mesophilic conditions of MAD and TPAD2 reactors, there was acetic acid mostly produced. For the TAD reactor, propionate was distinguished to be the most intensively produced during the Phases A and D, which could be a reason that methanogenesis was not so high as expected (Han *et al.*, 2020).

Comparing VFA production during the Phases A-B-C-D (Tables 3.7 and 3.8), it can be stated that the change of the stirring system did not influence that much in a long-term operation of both single- and double-stage reactors. An addition of the vertical paddle led to a slight enhancement of VFA production, except for TAD.

During Phase A, as at the most efficient in terms of methane production (Table 3.2), the highest content of acetic acid among other acids was monitored for MAD and TPAD2 – about 66% and 61%, respectively. Meanwhile, at the Phase D, the content of acetic acid at those AD systems was even higher: around 71% and 72%, respectively. Which goes along with lower methane production at the Phase D.

Propionic acid was detected at the level of maximum 21% for the MAD reactor at the Phase D and maximum of around 17% for TPAD2 at the Phase A. 55% of propionic acid out of all VFAs was detected only at the thermophilic conditions.

For the TPAD1 case, on average, almost 30% of acetic and 26% of propionic acid was measured at all experimental Phases.

It is important to mention that the VFA content should be in compliance with hydrogen production – hydrogen can be detected at lower propionate and higher acetate and butyrate indicating its main metabolic pathway (Kim *et al.*, 2013).

3.1.5 Hydrogen content

Indeed, the conditions formed in MAD and TPAD reactors looked more advantageous for hydrogen production over TAD system (see the subchapter "**3.1.4 VFA content**"). The lower standard deviation of hydrogen production indicates more stable hydrogen production at TPAD over time which can be considered as definite advantage of this AD system in terms of environmental sustainability, but not methane production efficiency.

Hydrogen content was quite significant at the Phase A (Table 3.9), whilst during Phase B and Phase D, it was either not detected at all, or negligible (not higher than 0.2% in both stages of TPAD system), apparently, due to the intensive mixing within the whole rector volume.

Table 3.9 – Hydrogen content in the biogas produced within the Phase A

experimental steps of Phase A	TAD (%)	MAD (%)	TPAD1 (%)	TPAD2 (%)

average values	1.58±1.39	4.68±1.01	8.38±3.12	2.68±0.88
maximum values*	5.46	5.49	11.93	3.07

Table 3.9 clearly underlines the period of the last two weeks of experiment when HRT multiplied three times passed: in the TAD reactor, hydrogen production raised up on more than 50%, meanwhile in the MAD reactor, it dropped on around 10%. However, even at the highest concentration in the thermophilic conditions, hydrogen content was lower on 40% than in the mesophilic conditions at the same period of time – within the last two weeks of the Phase A. Hydrogen content in both stages of TPAD system decreased in the last two weeks of experiment and became more stable according to the standard deviation of 0.9 and 0.6 for TPAD1 and TPAD2, respectively.

TPAD1 with its HRT of two days showed the most various hydrogen production values. However, in all AD systems during the Phase A, hydrogen production was either promoted by little mixing of medium. During the Phases B-C-D, it was either hindered by better mixing.

As the average pH values for each phase was not lower than 7.0 (Table 3.6), it can be said that there were never favourable conditions for hydrogen production as it was stated in (Cooney *et al.*, 2007; Kim *et al.*, 2013; Fu *et al.*, 2017) and defined to be in the range of 5.0-5.5, as the compulsory condition for hydrogen production promotion. Hence, at better mixing efficiency and pH of 7.0 and higher, there were no expectations for hydrogen detection in amounts higher than 0.2%.

3.1.6 Free ammonia and total ammonia nitrogen presence

The ammonia nitrogen concentration was measured and recalculated into free ammonia concentration as more in AD systems at each Phase of the laboratory experiments (Table 3.10) based on the pH data (Table 3.6).

Table 3.10 – Ammonia nitrogen and free ammonia content

Phase	Parameter	TAD	MAD	TPAD1	TPAD2
Phase A,	$N\text{-}NH_4$ (mg/L)	2513	2304	2452	2587
at HRT = 13.5 days	FA (NH_3) (mg/L)	24.7	25.4	7.5	42.9
Phase B,	$N\text{-}NH_4$ (mg/L)	3293	3084	3316	3299
at HRT = 13.5 days	FA (NH_3) (mg/L)	73.2	24.7	18.8	58.8
Phase C,	$N\text{-}NH_4$ (mg/L)	2437	2309	2140	2550
at HRT = 19.0 days	FA (NH_3) (mg/L)	60.6	24.9	13.0	67.8
Phase D,	$N\text{-}NH_4$ (mg/L)	3125	2474	3114	3005
at HRT = 13.5 days	FA (NH_3) (mg/L)	106.3	48.0	18.5	109.3

For the reason that organic matter is broken down under anaerobic conditions within a digester, anaerobic waste products like ammonium ions are being continuously produced. Ammonium ions are able to spontaneously react with hydroxide ions to ammonia and water.

When pH starts rising, more ammonium ions will react into ammonia. Concentrations of 150 mg/L of free ammonia, as more powerful inhibitor (Litav and Lehrer, 1978), and over 3000 mg/l of ammonia nitrogen can have serious inhibitory effects on anaerobic digestion (Yenigün & Demirel, 2013; Majd *et al.*, 2017). However, according to another literature source ((Gebreeyessus and Jenicek, 2016), ammonia in the range of 560-568 mgNH_3-N/l might lead to methanogenesis inhibition of 50% under thermophilic conditions. Therefore, monitoring ammonia nitrogen and free nitrous acid was crucial for taking into account when estimating methanogenic activity in all AD systems.

Even though the concentrations of free ammonia nitrogen do not exceed the claimed dangerous level when the methanogenesis is being hampered, ammonia nitrogen content is pretty high within the whole period of experiments (Table 3.6), but also below the potentiallly inhibitory concentrations.

3.1.7 Conclusions

In this part of the study, the stirring efficiency was investigated in AD systems with three different configurations regarding the methane production and some additional parameters such as hydrogen concentration in biogas and VFA content and pH value in digested sludge. Other parameters like suspension formation, temperature stability and foam formation were monitored.

CDF models were built based on operational data. The models visualised and supported the technological results.

With regards to the VS degradation efficiency and methane production, the Phase A with one-straight-blade impeller showed the best results. The AD reactors performed better at the Phase D with an additional insulation rather than at the Phase B. This confirms the necessity of proper insulation of AD reactor and its sensitivity to the heat fluctuations.

So as to VFA content, it varied significantly and reached the highest values in the fermenter (TPAD1), which is expected for a volumetric unit where the goal is an intensive hydrolysis and acetogenesis. Propionate was mainly produced in thermophilic conditions (up to 50%), acetate – in mesophilic ones (71% for MAD and 72% for TPAD2). In the TPAD1, the amount of different VFAs was different: both acetate and propionate were available at around 20-30%.

Hydrogen content was detected only at the Phase A when having only one-straight-blade paddle installed as a mixing equipment. Another important observation was that the highest concentrations were obtained in the last two weeks of the period for all reactors, with a maximum content in TPAD1 (6.2%) and minimum in TPAD2 (1.6%). Which means that hydrogen production may happen only at pretty low mixing or its absence.

In addition, several more findings were confirmed.

Mixing affects AD process in terms of following aspects: stirring speed, mixing regime, digester design including the design of the paddle. Mixing also affects AD process in both cases – single- and double-stage AD systems in the descending order: TAD > TPAD > MAD. TPAD system might be upgraded much better in the context of mixing equipment due to its double-stage configuration. The design of the paddle for each stage has to be defined experimentally and individually. This will allow to leverage the total methane production in TPAD.

In addition, relatively high methane content in the first stage of TPAD system was studied more in detailed from the microbiological point of view and compared to the microorganism consortium in TPAD2.

The majority of organisms were identified in digester, and it was concluded that the core community is similar in the most of AD systems. It was again confirmed that the separation of stages in the TPAD system configuration enhances the microbial diversity in the digesters. *Methanosarcina* (reaching even 12% of whole community) was dominant archaeal phylum in our study and contributed in methane production, similarly as in Danish full-scale digesters. Methanogens and acetogens were more abundant in TPAD2 than in the biofilm of TPAD1 and the least abundant in suspension in TPAD1, which was in good agreement with higher hydrogen content at TPAD1 than at TPAD2.

3.2 Digested sludge quality: Single-stage versus double-stage temperature-phased systems

3.2.1 Basic experimental results

As the experimental phase division in chronological order was introduced in the Table 2.4 (see the subchapter "**2.1.6 Experimental procedures**" of the Chapter 2 – Materials and Methods), there would be only the detailed description of the two Phases C and D at which the digested sludge quality was examined (Table 3.11).

Table 3.11 – The working parameter of AD systems during the Phases C and D

Type of reactor	Abbre-viation	Phase C		Phase D		Phases C and D
		HRT, days	Temperature range, °C	HRT, days	Temperature range, °C	Mixing speed, rpm**
Single, thermophilic	TAD	19.0	57±1.5°C	13.5	57±1.0°C*	50±1
Single, mesophilic	MAD	19.0	38±1.5°C	13.5	38±1.0°C*	50±1
Double-stage, thermophilic, (the first stage)	TPAD1	2.0	57±1.5°C	2.0	57±1.0°C*	30±1
Double-stage, mesophilic (the second stage)	TPAD2	17.0	38±1.5°C	11.5	38±1.0°C*	50±1

*Note 1: all digesters at the Phase D were covered with the insulation (Table 2.4)
**Note 2: all digesters at both Phases were continuously mixed at the fixed speed

The organic matter degradation is one of fundamental parameter of AD process efficiency. As the substrate characteristics changed during digester operation, the average values of VS and VSS (all in g/L) and removal efficiency were calculated for two different HRT tested – Table 3.12.

Table 3.12 – Organic matter removal within the Phases C and D

Phase	Parameter	TAD	MAD	TPAD1	TPAD2	Substrate
Phase C, at HRT = 19.0 days	Digested sludge VS (g/L)	30.8±1.7	31.3±2.6	35.4±4.1	28.6±1.0	42.3±4.1
	% VS removal (%)	27.2	26.0	16.3	32.4	-
Phase D, at HRT = 13.5 days	Digested sludge VS (g/L)	32.6±0.9	32.0±0.7	35.6±1.0	30.1±0.5	46.5±0.6
	% VS removal (%)	22.9	24.3	15.8	28.8	-

The achieved VS removal efficiency (23-32%) was relatively low, which reflects the fact that only thickened waste activated sludge was used as substrate, and that the systems were operated at a relatively high organic loading rate of 2.24-2.25 $kg.m^{-3}.day^{-1}$ (Phase A) and 3.58-3.62 $kg.m^{-3}.d^{-1}$ (Phase B) as a result of the relatively short HRT (Table 2.5). The similar VS removal rates (30-40%) were measured by Ge (2011) for

WAS as a substrate. Oppositely, Qin (2017) registered additional 8% of VS removal at TPAD compared to the conventional MAD. The results show that the VS removal efficiency decreased by only 2-5% after changing from 19 days (Phase A) to 13.5 days (Phase B) of HRT: 4.3% for TAD, 1.7% for MAD and 4.1% for TPAD. In terms of VSS, there was a slight removal rate increase of 1% for TAD which is negligible as the standard deviation was around the same value, a bigger removal rate increase of 4% for MAD and 4.8% for TPAD (Table 3.3). This means that shortening HRT reduced the degradation efficiency of all AD systems. However, acceptable efficiency was still achieved even at significantly shortened HRT, especially in TPAD. Fernandez-Rodriguez also stated the higher efficiencies for organic matter removal rate (30%) and methane production (26-60%) at TPAD than at any single-stage AD with the same HRT.

The operation of the TAD at short retention time was the least stable, which resulted in poor VS degradation efficiency and the accumulation of VFA (Table 3.7).

Such an improvement in organics' degradation could be mainly explained by addition of the insulation. The proof of it can be seen in the context of the batch SMA test results – Table 3.13.

Table 3.13 – Specific methane activity at both Phases C and D

Phase	Specific methane activity (gCH_4-COD.$gVSS^{-1}.d^{-1}$)			
	TAD	MAD	TPAD1	TPAD2
Phase C, HRT = 19.0 days	0.145±0.008	0.275±0.012	0.107±0.010	0.260±0.007
Phase D*,**, HRT = 13.5 days	0.262±0.010	0.242±0.009	0.141±0.011	0.300±0.008

*Note 1: the insulation added (Table 2.4)

**Note 2: at the Phase D, the vertical paddle was removed

The Table 3.13 shows that the methanogenic activity did not drop after changing from HRT = 19.0 days (Phase C) to HRT = 13.5 days (Phase D) and, moreover, it grew up to (4.9-11.1)% in terms of VSS.

Such an improvement in VSS solubilization could be explained by the improvement of insulation. It reduced the temperature fluctuations of the heating system – Table 3.11.

The Table 3.13 introduces the maximum specific methane activity of all studied sludges under correspondent conditions: the methanogenic sludge activity dropped only in MAD after the insulation was added. It can be explained by the fact that the Phase A took place during the late spring and summer when the ambient temperature in the laboratory was around (33-35) °C, and the AD temperature regime in both mesophilic reactors was maintained naturally, so that the improvement of the reactor heating system did not influence that much as it did under thermophilic conditions.

However, this action might not be the only one that affected the degradation that much. Additionally, good performance and sufficiently long laboratory installation operation period (more than a year of constant operation), the AD systems were successfully operated also at shorter HRT.

In order to have a comprehensive picture, it is important to evaluate not only the degradation efficiency in terms of VS and VSS (Table 3.12), but also balance between the degradation efficiency and methane production rate. The results in methane production are depicted in the Table 3.2.

The double-stage TPAD system achieved the highest specific methane production in both periods: 0.23 L/g COD_{added} at the Phase C vs. 0.17 L/g COD_{added} at the Phase D (Table 3.2). At the TAD system: 0.17 (Phase C) vs. 0.12 (Phase D) L/g COD_{added}. At the MAD conditions: 0.16 (Phase C) vs. 0.13 (Phase D) L/g COD_{added}. Indeed, the TPAD system reached comparable results with HRT of 13.5 days (0.17 L/g COD_{added}) to TAD and MAD with HRT of 19 days (0.17 and 0.16 L/g COD, respectively). It is interesting that, according to the statistical analysis performed, the difference in methane production at both Phases is statistically significant for all AD systems, besides TAD-MAD at the Phase D (HRT = 13.5 days). The correlations were considered statistically significant at a 95% confidence interval ($a < 0.05$). Amodeo (2021) also proved that TPAD showed better performnace in terms of methane production up to 20% in comparison to the single-stage MAD.

The double-stage TPAD system in principle separates the AD stages: hydrolysis and acidogenesis take place in the 1st stage, while acetogenesis and methanogenesis occur in the 2nd stage (Cao et al., 2020). Therefore, the second stage of the TPAD (TAPD2) was expected to have the highest methane content in biogas – Table 3.14.

Table 3.14 – Biogas composition

Phase	TAD (%)		MAD (%)		TPAD1 (%)		TPAD2 (%)	
	CH_4	CO_2	CH_4	CO_2	CH_4	CO_2	CH_4	CO_2
Phase C, at HRT = 19 days	**61.7**±4.8	34.7±5.0	**66.1**±1.8	30.2±3.6	58.9±12.8	33.2±8.4	**70.9**±2.7	24.7±2.7
Phase D, at HRT = 13.5 days	**61.7**±1.7	33.5±2.3	**64.2**±2.1	29.9±1.6	53.4±4.7	39.8±5.0	**71.4**±1.3	23.8±0.9

Besides, the methane content in TPAD1 was expected to be much lower because of the very short retention time (2.0 days) According to the literature, the generation time of methanogens may vary a broad range of 0.1-12.4 days (Wu *et al.*, 2020). In this case, the presence of methanogens can be explained by the production of biofilm on the digester walls and the mixing device. To our best knowledge, much higher retention time of biofilm in comparison with suspended biomass allowed for an accumulation

of methanogens inside the digester, as the HRT of TPAD1 of 2 days is not enough to avoid washing out the methanogens (Qin *et al.*, 2017). However, the presence of fast-growing methanogens (generation time 4–12 hours) cannot be ruled out either (Weimer, 1998). Especially when any of the other means for methanogenic inhibition like lowering pH and dosing methanogenic inhibitors were not performed (Ruffino *et al.*, 2020).

Our experimental results suggest that the TPAD system is beneficial due to improved hydrolysis and acidogenesis in the first stage and optimized conditions for methanogenesis in the second stage. Such a system seems to be sufficiently efficient mainly at a short total HRT of TPAD up to 14 days, which can reduce the footprint and investment costs. Xiao (2018) stated HRT as the crucial parameter that can influence on efficiency of AD, and HRT of 30 days puts all types of AD become more or less same in terms of biogas production which makes more energy demanding TAD and TPAD less economically interesting. Bolzonella (2007), Wu (2015) and Amodeo (2021) underlined that the first stage of TPAD was the most efficient at 2-3 days, when the total HRT was less than 20 days.

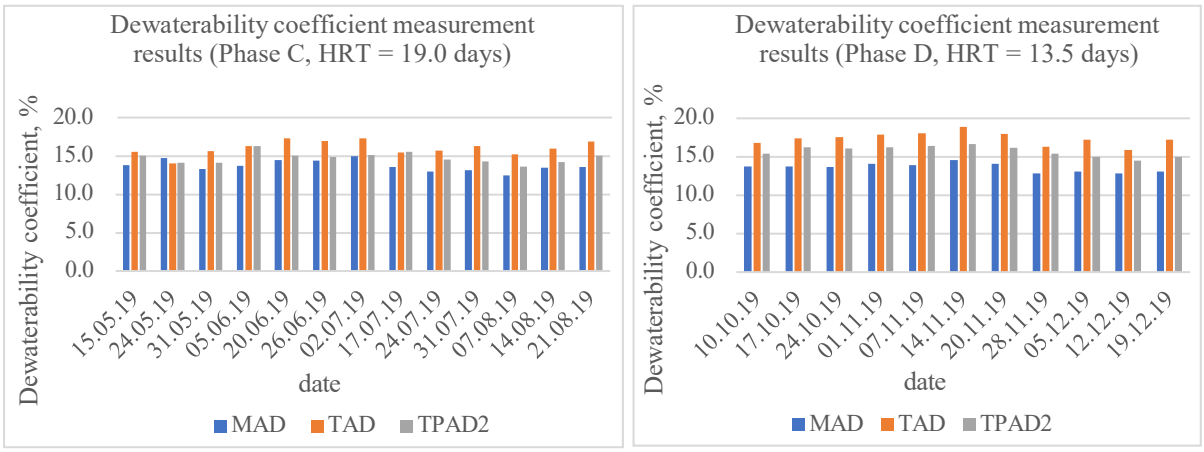
3.2.2 Centrifugation

Centrifuging as one of the dewaterability measurement methods was applied to all types of digested sludge produced within both Phases.

The dewaterability of the digested sludges from TAD, MAD and TPAD systems was determined by means of a dewaterability coefficient, which allows for assessing the concentration of dry matter in a dewatered digested sludge sample. Thus, the higher dewaterability coefficient, the better dewatering efficiency (Table 3.15, Figure 3.9).

Table 3.15 – Dewaterability coefficient at both Phases

Phase	Dewaterability coefficient, %		
	TAD	MAD	TPAD
Phase C, at HRT = 19 days	16.1±0.9	13.8±0.7	14.8±0.7
Phase D, at HRT = 13.5 days	17.4±0.9	13.6±0.6	15.7±0.7



(a) (b)

Figure 3.9 – Dewaterability coefficient at the Phase C and D

It was found that the difference among dewaterability coefficients was relatively small, but still statistically significant among all types of AD systems at both Phases. Hence, the best dewaterability was determined for the digested sludge from TAD, followed by TPAD, and MAD. Furthermore, decreasing the HRT from 19 to 13.5 days did not decrease the dewaterability, in fact, it was slightly increased for TAD and TPAD. This is in accordance with the statement that the higher organic matter content hinders the dewatering ability of WAS (Wang *et al.*, 2020).

Specifically, at 19 days of HRT, the digested sludges dewaterability was 13.8%, 14.8% and 16.1% for the MAD, TPAD and TAD, respectively; while at 13.5 days of HRT, the digested sludges dewaterability was 13.6%, 15.7% and 17.4% for the MAD, TPAD and TAD, respectively (Table 6). So, the dewaterability of TAD was 9.8% and 8.1% higher than TPAD at 19 and 13.5 days of HRT, respectively, while the dewaterability of TAD was 21.8% and 14.3% higher than MAD at 19 and 13.5 days of HRT, respectively.

Hence, despite just a slight effect of HRT change from 13.5 to 19 days on all types of AD digested sludge dewaterability, the digested sludge from TAD showed continuously the better performance concerning the ability to "loose" the water under the centrifugal forces. The worst quality of the digested sludge after MAD can be explained by lower degradability of the sludge in terms of VS and VSS (Tables 3.12 and 3.3).

3.2.3 Mechanical pressing

To our best knowledge, the sludge cake concentrations obtained by the mechanical pressing method are in a good agreement with the range of results generally achieved in full-scale wastewater treatment plants (Toutian *et al.*, 2020, Cai *et al.*, 2021). The ratio between the wet sample and dry cake weight shows how much the digested sludge can be dewatered. The results of mechanical pressing are depicted in the Table 3.16.

Table 3.16 – The results of mechanical pressing

Phase	Parameter	TAD	MAD	TPAD2
Phase C, at HRT = 19.0 days	TS of sludge cake (%)	25.0±1.0	26.1±3.8	25.6±1.7
	Polymer dose (g/kgTS)	35.0	35.0	35.0
Phase D, at HRT = 13.5 days	TS of sludge cake (%)	30.8±4.2	31.4±2.4	28.7±4.3
	Polymer dose (g/kgTS)	30.0	30.0	32.5

In agreement with centrifugation results (Table 3.15, Figure 3.9), the digested sludge dewaterability did not decrease with the HRT. In fact, it was slighlty improved after decresing the HRT from 19 to 13.5 days (Table 3.16). In addition, the optimal dose of flocculant was slightly lower at the shorter HRT: 35 vs. 30-32.5 g/kgTS for 19 and 13.5 days of HRT, respectively. However, statistically, the obtained results turned out to be insignificant.

What is also important to mention that though TAD digested sludge showed relatively the same results of dewaterability as MAD and TPAD did, the amount of mechanical pressing test failures was the highest: 3 positive results out of 10 tests for TAD; 10 out of 10 for MAD and 7 out of 10 for TPAD. That should be a primarily estimation of AD sludge exposed to any dewatering method. This means that TAD digested sludge was the least prone to be dewatered.

The results of different dewaterability measurement methods were quite different, which goes along with the literature (Svennevik *et al.*, 2019). However, the trend was similar to another study where TAD digested sludge showed a better ability to be dewatered, and demanded higher flocculant consumption (Lloret *et al.*, 2013).

A way dewaterability influences a final disposal is straightforward: it is always better, when it is as high as possible, as by removing the contained water the sludge got reduced in volume, which is beneficial at least in transportation expenses and any following final disposal starting from old-fashioned landfilling and heading to its reuse in road construction via incineration or direct usage in agriculture (Liu *et al.*, 2021; Wei *et al.*, 2018; Wu *et al.*, 2020).

3.2.4 Lower calorific value calculation and dewaterability estimation based on elemental analysis

The difference in digested sludge quality can be characterized by elemental analysis as well. The average results of elemental analysis are shown in the Table 3.17.

Table 3.17 – The elemental composition of digested sludge

Phase	Element, %	TAD	MAD	TPAD1	TPAD2	Substrate
	N	3.87±0.05	4.23±0.21	4.31±0.05	3.83±0.22	5.37±0.13

Phase C, at HRT = 19.0 days	C	25.70±0.27	25.56±0.25	27.32±0.70	24.60±0.19	30.26±0.50
	H	4.39±0.14	4.36±0.12	4.65±0.11	4.29±0.06	4.97±0.19
	S	0.85±0.02	0.84±0.01	0.84±0.01	0.88±0.04	0.77±0.01
	O	65.20±0.48	65.01±0.59	62.88±0.87	66.41±0.51	58.64±0.82
Phase D, at HRT = 13.5 days	N	4.50±0.06	5.18±0.17	5.06±0.07	4.61±0.07	6.50±0.03
	C	30.75±0.53	30.45±0.34	32.58±0.07	29.80±0.59	35.28±0.30
	H	4.77±0.21	4.74±0.18	4.95±0.23	4.63±0.27	5.31±0.20
	S	0.92±0.03	0.98±0.01	0.90±0.03	0.99±0.01	0.81±0.01
	O	59.06±0.79	58.66±0.28	56.52±0.35	59.97±0.80	52.10±0.50

Furthermore, the lower calorific value (LCV) was calculated according to the literature source (Nzihou *et al.*, 2014) and assessed in the context of the initial value of substrate LCV – Table 3.18.

Table 3.18 – Lower calorific value of the digested sludges and substrate

Phase	Parameter	TAD	MAD	TPAD1	TPAD2	Substrate
Phase C, at HRT = 19.0 days	LCV (kJ/kg)	9,157±76	9,111±73	9,742±251	8,750±63	10,827±155
	drop in LCV (%)	15.4	15.8	10.0	19.2	-
Phase D, at HRT = 13.5 days	LCV (kJ/kg)	11,127±97	10,949±137	11,715±29	10,793±89	12,783±8.9
	drop in LCV (%)	13.0	14.3	8.4	15.6	-

During the AD process, part of the substrate organic matter content is biodegraded and converted into methane, thus reducing the lower the energy content of the sludge, here determined by the LCV (Menon *et al.*, 2020). The highest LCV decrease was observed in the TPAD (around 19% with HRT of 19 days), which supports the highest rate of organics transformation into biogas. In addition, according to statistical test ANOVA, it was assessed that obtained LCV data were significantly different only at the Phase A (HRT = 19.0 days) and in between TAD-TPAD and MAD-TPAD. That went along with the data on VS removal rate (Table 3.12): 32.4% of VS removal at TPAD against 27.2% at TAD and 26.0% at MAD. The same trend was observed regarding the methane production (Table 3.2): 0.23 L/g COD_{added} at TPAD vs. 0.17 L/g COD_{added} at TAD and 0.16 L/g COD_{added} at MAD. Which brings us to two interesting hypotheses: (1) the longer HRT – the bigger difference among the introduced AD systems; (2) the longer HRT – the bigger difference between single- and double-stage AD systems.

Considering the sludge cake concentration presented in Table 3.16, it can be stated that, despite the left water content, the real calorific value (related to the wet sludge cake after dewatering) remained quite high which is important especially when thermal treatment is applied as final treatment process. As it is known, according to

Tanner' triangle, the autothermic process of combustion is highly dependent of the fuel LCV and possible unless LCV of the digested sludge is lower than 50% of loss in LCV (Flaga, 2010).

It was reported that elemental analysis of the sludge can be also used for the prediction of dewatered sludge cake TSS concentration (Svennevik *et al.*, 2019). The results depicted in the Table 3.19 have certain extent of correlation with the solids content of digested sludge samples after mechanical pressing shown in the Table 3.18.

Table 3.19 – Sludge cake solids prediction for the digested sludge after the conventional AD and its correlation with mechanical pressing results

Phase	Parameter	TAD	MAD	TPAD	Substrate
Phase C, at HRT = 19.0 days	cake solids (TSS) (%)	26.5	23.8	25.6	20.4
	correl. coef.* (-)	1.06	0.91	1.00	-
Phase D, at HRT = 13.5 days	cake solids (TSS) (%)	23.6	20.6	22.9	16.0
	correl. coef.* (-)	0.77	0.66	0.80	-

Note: *correl.coef. – correlation coefficient between the mechanical pressing results (Table 3.16) and sludge cake solids concentration introduced by Svennevik (2019)

It was noted that at the Phase C (HRT = 19.0 days) the correlation coefficient was around 1.0 for all AD systems. At the Phase D (HRT = 13.5 days), the correlation coefficient was on approximately 20% lower than for the correspondent AD system. It shows that at longer HRT the theoretically calculated prognosis on sludge cake solids concentration closer (±10%) to the experimental results of dewatering process by mechanical pressing than at the shorter HRT (lower on 20-30%, on average). This means that at HRTs shorter than 19.0 days, the calculated results on sludge dewaterability properties and based on elemental analysis (EA) should be verified by laboratory experiments. There might have been obtained better actual results than anticipated by theoretical calculations.

3.2.5 Hygienization efficiency assessment

It is known that sewage sludge contains different types of pathogens including eggs of parasitic worms, bacteria, and viruses. AD is one of the effective methods for the reduction of pathogens to allow the application of digested sludge for agriculture safely (Seleiman et al., 2020). However, depending on the temperature regime, the results of hygienization may vary: after MAD, the digested sludge does not meet the requirements that would permit to apply the digested sludge as fertilizer to the soil, meanwhile, after TAD, the digested sludge possesses the higher pathogenic safety

results (Hupfauf *et al.*, 2020). Thus, normally, the TAD digested sludge meets the requirements of Class A biosolids, which is not feasible for the MAD (Lloret *et al.*, 2013).

Microbiological analyses were performed to evaluate the potential of digested sludge to be applied on agricultural fields, directly or after a post-treatment step, which is one of final disposal application of digested sludge (Baba *et al.*, 2013) – Table 3.20.

Table 3.20 – Microbiological characterization of the digested sludge concerning the pathogenic safety

Phase	microbiological parameter	WAS from the feeding bucket, stored at +11.5 ºC	TAD	MAD	TPAD1	TPAD2
Phase C, at HRT = 19.0 days	micro-organisms cultivated at 22 °C*, CFU/g	2.5×10^{6}	2.1×10^{4}	6.2×10^{4}	3.7×10^{4}	1.3×10^{5}
	micro-organisms cultivated at 36 °C*, CFU/g	1.2×10^{6}	1.4×10^{4}	7.4×10^{4}	3.3×10^{4}	9.1×10^{4}
	COLI*, CFU/g	8.2×10^{4}	**<1**	**299**	**<1**	**38**
	*E. coli**, CFU/g	4.9×10^{4}	**<1**	**155**	**<1**	**<1**
	CLO*, CFU/g	2.3×10^{4}	$\mathbf{2.5}\times10^{3}$	$\mathbf{1.3}\times10^{4}$	$\mathbf{1.5}\times10^{4}$	$\mathbf{4.8}\times10^{3}$
Phase D, at HRT = 13.5 days	micro-organisms cultivated at 22 °C*, CFU/g	2.8×10^{7}	1.4×10^{5}	1.2×10^{6}	4.0×10^{5}	1.2×10^{6}
	micro-organisms cultivated at 36 °C*, CFU/g	1.2×10^{7}	2.2×10^{5}	9.8×10^{5}	9.7×10^{5}	1.7×10^{6}
	COLI*, CFU/g	3.7×10^{4}	**<1**	**38**	**<1**	**<1**
	*E. coli**, CFU/g	2.0×10^{3}	**<1**	**20**	**<1**	**<1**
	CLO*, CFU/g	5.0×10^{4}	$\mathbf{1.4}\times10^{3}$	$\mathbf{1.8}\times10^{4}$	$\mathbf{9.2}\times10^{3}$	$\mathbf{4.9}\times10^{3}$

Note: *CFU – colony forming units, TC22 °C – total counts of culturable microorganisms at 22 °C; TC36 °C – total counts of culturable microorganisms at 36 °C, COLI – total counts of coliforms, ECOLI – total counts of *Escherichia coli*, CLO – total counts of *Clostridium perfringens*

The Table 3.20 shows that both digestion systems using thermophilic conditions outperform the mesophilic one. Concerning the mesophilic conditions, the reduction of pathogenic bacteria was less efficient. Decreasing the HRT from 19 to 13.5 days did not impair the pathogenic safety in all evaluated AD systems, since the results could be even better.

The statistics revealed that the only significant difference in microbiological tests was observed for the Phase C with 19.0 days of HRT regarding two microbiological parameters of coliforms and *Escherichia coli* and only in relation of TAD and TPAD towards MAD. The difference between TAD and TPAD was insignificant. TPAD achieved only slightly worse results in comparison with the TAD, however, the hygienization was sufficient for the application of digested sludge to the soil only for the case of the Phase C with 19.0 days of HRT. It was also noticed that though the first stage of the TPAD under thermophilic conditions showed a number of coliforms and *Escherichia coli* below detection level, after changing to mesophilic conditions in the second stage, they appeared again. Which might be of concern when defining the HRT of each stage of the double-stage AD system. However, in the TAD digested sludge, as well as in the TPAD digested sludge pathogens were present in significantly lower amounts than after the MAD process. This goes along with the results obtained by Fu (2014), which say that after 2 days of the fermenter HRT under thermophilic conditions some pathogens were not detected, and after 3 days of the fermenter HRT *Escherichia coli* was completely deactivated. This goes along with the results obtained by Fu (2014), which say that after 2 days of the fermenter HRT under thermophilic conditions some pathogens were not detected, and after 3 days of the fermenter HRT *Escherichia coli* was completely deactivated.

The assured pathogenic safety of TAD digested sludge and the sludge obtained after the TPAD system with HRT of the fermenter long enough for full deactivation of faecal indicators allows to apply the sludge to direct use in agriculture (Seleiman *et al.*, 2020).

3.2.6 Comparison of results

All the data obtained was evaluated and put into the Table 3.21 for better assessment.

Table 3.21 – Comparison of the obtained data concerning TAD, MAD and TPAD

Phase	Parameter	TAD	MAD	TPAD1	TPAD2	TPAD
Phase C, at HRT = 19.0 days	VS removal (%)	+	-	ND	++	ND
	VFA concentration (mgCOD/L)	+	++	-	++	ND
	Methane production ($mL/gCOD_{added}$)	++	++	-	++	+++
	Dewaterability coefficient (%)	++	-	ND	+	ND
	Polymer dose (g/kgTS)	-	-	ND	-	ND
	LCV (kJ/kg)	+	+	ND	-	ND
	cake solids (TSS) (%)	++	-	ND	+	ND

	microorganisms cultivated at 22 °C (CFU/g)	+++	+	++	-	ND
	microorganisms cultivated at 36 °C (CFU/g)	+++	+	++	-	ND
	COLI (CFU/g)	+	-	+	+	ND
	E. coli (CFU/g)	+	-	+	+	ND
	CLO (CFU/g)	+	-	-	+	ND
	WWTP-LCA*	+	-	ND	ND	++
	SL-LCA**	++	+	ND	ND	0
Phase D, at HRT = 13.5 days	VS removal (%)	-	-	ND	+	ND
	VFA concentration (mgCOD/L)	+	++	-	++	ND
	Methane production ($mL/gCOD_{added}$)	++	++	-	++	+++
	Dewaterability coefficient (%)	++	-	ND	+	ND
	Polymer dose (g/kgTS)	-	-	ND	+	ND
	LCV (kJ/kg)	+	+	ND	-	ND
	cake solids (TSS) (%)	++	-	ND	+	ND
	microorganisms cultivated at 22 °C (CFU/g)	++	-	+	-	ND
	microorganisms cultivated at 36 °C (CFU/g)	++	+	+	-	ND
	COLI (CFU/g)	-	-	-	-	ND
	E. coli (CFU/g)	-	-	-	-	ND
	CLO (CFU/g)	+++	-	+	++	-

Note: "-" – the worst result of all); "+", "++", "+++" - relative estimation in comparison to the worst result (the more "+" – the better results compared to "-"-result); ND – no data

*WWTP-LCA – life cycle assessment of each AD system analysed separately as a part of the whole WWTP with the functional unit of 1 m^3 of treated wastewater (performed only for the Phase C, HRT – 19.0 days) (Lanko *et al.*, 2020)

**SL-LCA – life cycle assessment of each AD system analysed separately as an AD system only with the functional unit of 1 m^3 of produced methane (performed only for the Phase C, HRT – 19.0 days) (Lanko *et al.*, 2020)

When considering the Table 3.21, all the measured parameters can be split into 6 groups (for the Phases C and D altogether, as the was a negligible difference between the Phases): (1) organic matter degradation efficiency and methane production; (2) process stability (VFA content); (3) sludge quality (dewaterability); (4) final disposal as a fuel (LCV); (5) final disposal as a fertilizer (microbiological parameters); (6, for the

Phase C only, as the LCA was performed only at HRT of 19 days (Lanko *et al.*, 2020) environmental burden (LCA). The results are depicted in the Table 3.22.

Table 3.22 – Final comparison of TAD, MAD and TPAD

Phase	Parameter	TAD	MAD	TPAD
Phases C and D	Degradation efficiency and methane production	2	3	1
	Process stability (VFA content)	2	1	1
	Digestate quality (dewaterability)	1	3	2
	Final disposal as a fuel (LCV)	1	1	2
	Final disposal as a fertilizer (pathogen safety)	1	3	2
	5-group average value	1.40	2.20	1.60
Phase A	Environmental burden	1	3	2
	6-group average value	1.33	2.33	1.67

Note: "1" – one point which relates to the best result; "2" – two points mean the middle-point result; "3" – three points mean the worst result

Based on the Table 3.22, it can be stated that at both Phases and, correspondently, at both HRTs of 19 and 13.5 days, TAD outperformed. Additionally included LCA estimation (Lanko *et al.*, 2020) allowed TAD to get more "points" and improve the final mark from 1.40 to 1.33. The difference according to the 5-group-parameter averages, TAD got 0.2-point advantage over TPAD, and TPAD got the 0.6-point advantage over MAD, which resulted in difference between TAD and MAD up to 0.8. Looking at 6-group average values, it can be claimed that the results were even more improved for TAD and worsened for TPAD and MAD. TAD advantage over TPAD grew up to 0.34 points, and TPAD advantage went up to 0.66 points; overall difference between TAD and MAD went up to 1.0.

It is important to mention that the Table 3.22 represents quite rough estimation, as only main characteristics of AD process were compared.

In addition, each characteristic of AD has a different value economically- and ecologically- wise, which has to be considered when making a choice of AD system for implementation at each WWTP individually. Hence, there could be presented a bigger number of groups, and, in its turn, each group (including the introduced ones) could contain more AD parameters. Nevertheless, it gives a good overview of single- and double- stage AD systems according to main process characteristics specifically grouped according to the total AD efficiency, its stability, digestate quality and its possible final disposal.

The obtained data can be compared with the data published earlier – Table 3.23.

Table 3.23 – Comparison of TAD, MAD and TPAD results with other studies

Phase	Parameter		TAD	MAD	TPAD	other source
Phases C and D	Degradation efficiency as VS decrease (%)	study	22.9-27.2	26.0-32.8	28.8-32.4	-
		other sources	-	24-34	38-48	[40]

Specific methane production (mL/gVS_{dded})	study	168-244*	189-220*	314-413*	-
	other sources	-	111-185	370	[40]
			140	360	[19]
Process stability as VFA content (mgCOD/L)	study	3.9-4.8	0.3-0.5	0.4-0.5	-
	other sources	0.87**	0.16**	0.31**	[45]
Energetic value as LCV loss (kJ/kg)	study	13.0-15.4	14.3-15.8	15.6-19.2	-
	other sources	16.24**	16.74**	16.59**	[45]

Note: *The average values of specific methane production are recalculated to gVS_{added} based on data in the Table 2.
**The data presented in the literature source relate to the food waste, not sewage sludge.

In addition to the Table 3.23 data, it is needed to mention that [54, 55, 56] stated that TPAD process with 15 days of HRT outperforms any of single-stage systems in terms of dewaterability, though there are still many unsettled issues about sludge dewaterability measurement and assessment [32]. The same is valid concerning pathogenic safety, with the only exceptional requirement of minimum HRT of the 1st stage which should be equal to 3 days. Hence, these studies indicate that the TPAD seems to be the most beneficial alternative among other AD systems at short HRT, similarly as in the presented study.

3.2.7 Conclusions

Based on the results obtained in this study, the following conclusions can be drawn.

1. Organic matter removal and methane production experimental data clearly showed that TPAD got the best results, then followed by TAD and, finally, by MAD.
2. Regarding the dewaterability, the results varied depending on the physical mechanism of the dewatering test. By centrifugation without flocculant addition, the highest dewaterability was obtained by TAD, which was 8.1% and 9.8% higher than TPAD and 14.3% and 21% higher than MAD during both HRT (13.5 and 19.0 days, respectively). The mechanical pressing results showed the statistical insignificance among the AD systems.
3. The calorific value of the sludge was reduced by 19.2% after the TPAD at the Phase C with HRT of 19 days which was the only statistically significant difference between TPAD and TAD/MAD. At the Phase D with HRT of 13.5, any AD system did not show any statistical difference in relation to the other AD systems.
4. Deactivation of pathogens was proven for the TAD digestate regardless of the HRT, but not by the MAD digested sludge, while TPAD showed different results depending on HRT. It seems that HRT of the first stage of TPAD is crucial in relation of TPAD digestate pathogenic safety. Hence, it might still be a concern the possibility of using TPAD digestate directly for agricultural purposes.

To sum up digested quality evaluation, several sludge properties were quantified and compared to aggregate the data for making a decision about the suitability of different

sludge types for different sludge valorisation routes. It was shown that the TAD digestate can be applied directly in agriculture, while the TPAD digestate might also be used as a fertilizer successfully depending on fermenter HRT assuring the pathogenic safety. With the highest absolute value of LCV (for dry sludge), MAD is the best for being used as a fuel preserving higher portion of organic matter not transformed into biogas but losing this advantage due to the worse dewaterability in comparison with TAD and TPAD. In terms of environmental burden, TAD turned out to be the most environmentally friendly one, followed by TPAD and MAD.

In agreement with other studies, it can be said that double-stage TPAD is the most beneficial AD system among the others allowing a flexible sludge valorisation in different ways. However, its output is highly dependent on: (1) the AD substrate and its characteristics; (2) the properly selected operating parameters such as temperature regime, HRT, and OLR.

3.3 Life cycle assessment of the mesophilic, thermophilic and temperature phased anaerobic digestion of sewage sludge

3.3.1 WWTP-LCA with mesophilic, thermophilic or temperature-phased anaerobic digestion

The LCA tool was applied to compare three different AD systems, namely, TAD, MAD and TPAD. Data on the operation and performance of these systems treating the same sewage sludge was gathered from three lab-scale digesters (Tables 2.5, 3.11, 3.12, 3.16, 3.18) which were run during five months of the Phase C (Table 2.4).

The results of the LCA for the whole WWTP (WWTP-LCA) are shown in the Figure 3.10.

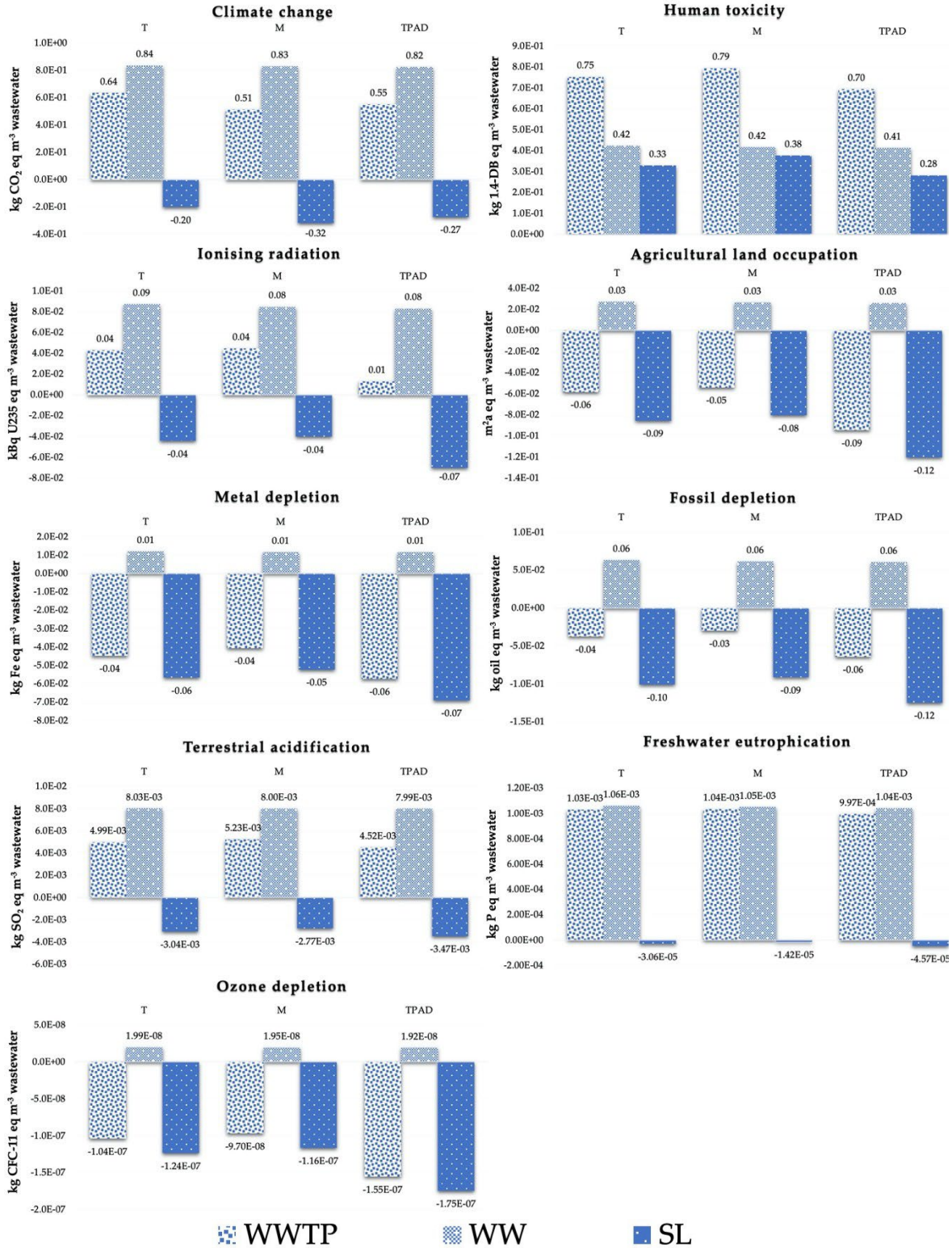


Figure 3.10 – Potential environmental impacts for the three scenarios of the whole WWTP (WWTP-LCA): TAD, MAD and TPAD. WWTP: wastewater treatment plant; WW: wastewater line; SL: sludge line. Results shown for the FU: 1 m^3 of treated wastewater

The Figure 3.10 includes all environmental impact categories considered in this study, and within each impact category there are three scenarios: mesophilic, thermophilic and temperature-phased ones. For each scenario, results are shown for the whole

WWTP, and separately for the wastewater treatment line and the sludge treatment line; this disaggregation of results is done to better identify the contributions of each process stage to the overall impacts. Positive values represent the environmental impacts, while negative values refer to the avoided environmental impacts.

According to the results (Figure 3.10), the differences among the three AD scenarios are not significantly large, however there are some trends that are here discussed. First, the wastewater treatment line would lead to larger environmental impacts than those cases where the sludge line is incorporated into the AD system. Thus, any implemented AD improves the environmental status of the WWTP mainly due to the credits obtained from the substitution of electricity generation from the fossil fuels (Bacenetti & Fiala, 2015). Similar results have been reported for other LCA studies on full-scale AD plants (Yu *et al.*, 2020).

In general terms, TPAD has the lowest environmental impacts, in comparison to TAD and MAD, for eight out of the nine impact categories presented in Figure 3.13, except for Climate change. Furthermore, a one-to-one comparison between TAD and MAD shows that their calculated environmental impacts are virtually the same for five out the nine compared categories (*i.e.* Ionising radiation, Agricultural land occupation, Metal depletion, Fossil depletion, and Freshwater eutrophication), and with a slightly better environmental performance (meaning lower environmental impacts) for TAD over MAD in two impact categories (*i.e.* Terrestrial acidification and Ozone depletion). TAD outperforms both MAD and TPAD in one category (*i.e.* Climate change), and has a slightly better performance than MAD in only one category (*i.e.* Human toxicity).

In the case of Climate change, the biggest impacts are caused by the wastewater treatment line (Figure 3.13). Conversely, the sludge line decreases the Climate change impacts up to 38% for MAD, around 35% for TPAD and 24% for TAD. Climate change is related to non-renewable energy consumption, which is especially high in the biological reactor of activated sludge WWTP, accounting for more than 50% of the total energy consumption – Table 2.6 and (Lizarralde *et al.*, 2018). The positive influence of sludge line mainly comes from AD which supplies with the fertilizer obtained after WAS is digested (Sludge disposal_SL) that substituted the industrial production of the fertilizer with its harmful effect through Climate change. Additionally, AD generates renewable energy out of the biogas produced as a result of the organic matter biodegradation, and counter-balances non-renewable energy consumption that would otherwise be required to fuel the process. The highest avoided impacts on Climate change are obtained with the MAD which is 40% and 42% better than TAD and TPAD, respectively. These avoided impacts occur due to a type of digested sludge disposal (which is composting and consequent agricultural land application for MAD, agricultural land application alone – for TAD and TPAD) and to its larger amount in comparison to TAD and TPAD (Table 2.5). In terms of the energy balance, TPAD is more beneficial than TAD and MAD by more than 50%

within the sludge line. The lowest total avoided impact on Climate change is obtained with the thermophilic digestion, as a consequence of the energy balance of the process, *i.e.* energy produced vs. energy consumed by each AD system – Figure 3.11.

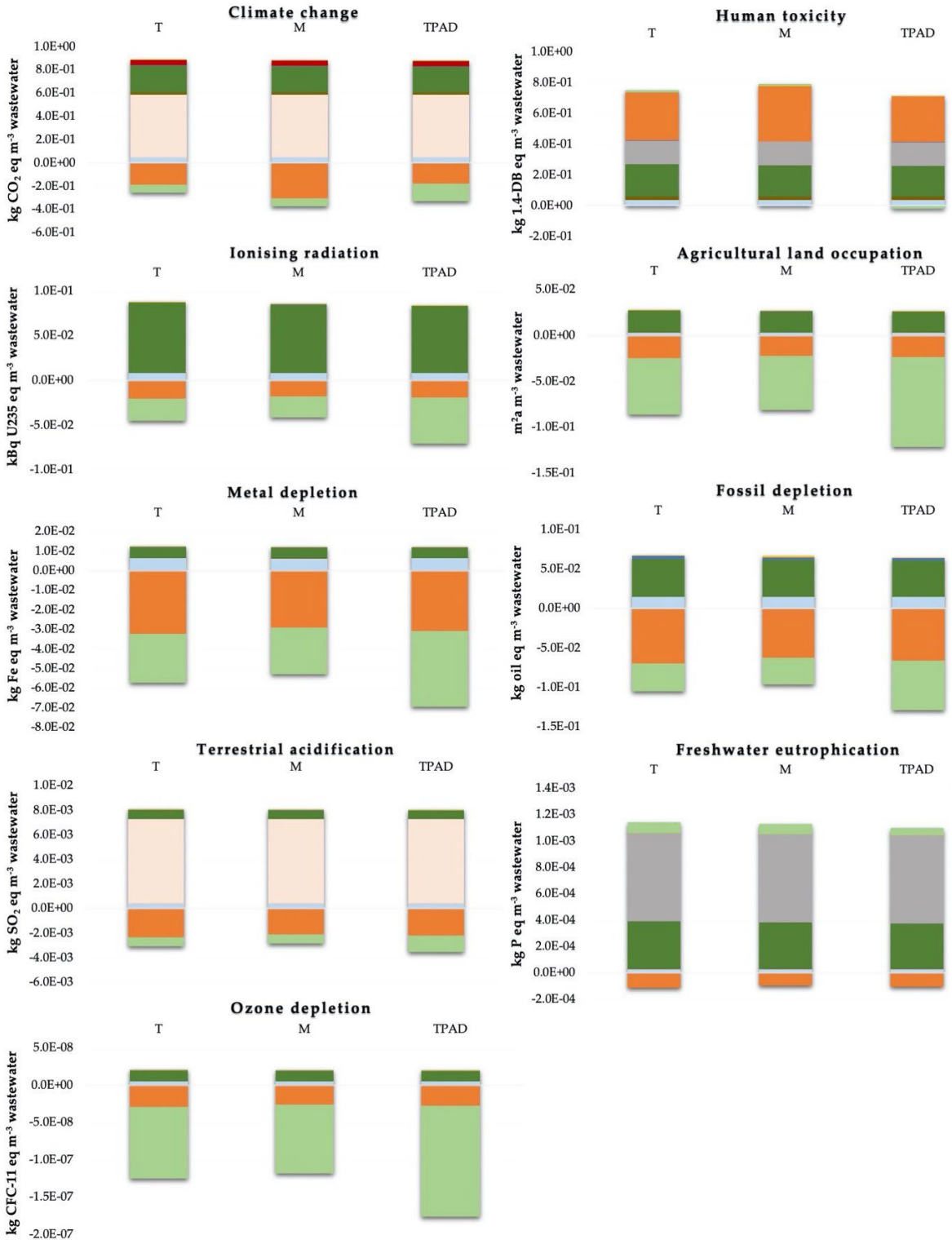


Figure 3.11 – Contribution analysis of the potential environmental impacts for the three scenarios of both wastewater and sludge lines (WWTP-LCA): TAD, MAD, TPAD. Results shown for the FU: 1 m^3 of wastewater treated

In the case of Climate change, the biggest impacts are caused by the wastewater treatment line (Figure 3.11). Conversely, the sludge line decreases the Climate change impacts up to 38% for MAD, around 35% for TPAD, and 24% for TAD. Climate change is related to nonrenewable energy consumption, which is especially high in the biological reactor of activated sludge WWTP, accounting for more than 50% of the total energy consumption – Table 2.6 and (Lizarralde *et al.*, 2018). The positive influence of sludge line mainly comes from AD which supplies with the fertilizer obtained after WAS is digested (Sludge disposal_SL) that substituted the industrial production of the fertilizer with its harmful effect through Climate change. Additionally, AD generates renewable energy out of the biogas produced as a result of the organic matter biodegradation, and counterbalances nonrenewable energy consumption that would otherwise be required to fuel the process. The highest avoided impacts on Climate change are obtained with MAD which is 40% and 42% better than TAD and TPAD, respectively. These avoided impacts occur due to a type of digested sludge disposal (which is composting and consequent agricultural land application for MAD, agricultural land application alone—for TAD and TPAD) and to its larger amount in comparison to TAD and TPAD (Table 2.5). In terms of the energy balance, TPAD is more beneficial than TAD and MAD by more than 50% within the sludge line. The lowest total avoided impact on Climate change is obtained with the thermophilic digestion, as a consequence of the energy balance of the process, i.e., energy produced vs. energy consumed by each AD system. This is reflected in the results of WWTP-LCA regarding all constituents are depicted in Figure 3.11.

With regard to the factors that contributed the most to the environmental impact of Climate change, those emissions to the air (Air emissions_WW) and from the energy demand (Electricity_WW) in the wastewater treatment line are the most significant ones (i.e., around 60% and 25%, respectively, of all contributors from the wastewater treatment line). The contribution from the emissions to the air in the sludge line (Air emissions_SL) are only 5% (Figure 3.11). Hence, the total environmental impact was partly compensated by land application as the final sludge disposal (Sludge disposal_SL) and energy produced from the methane (Electricity_SL) obtained during AD with the following percentage of these two factor contributions, respectively: 72% and 28% for TAD, 82% and 18% for MAD, and 54% and 46% for TPAD. The balance of these two factors for TPAD shows better long-term performance of this AD technology.

For Human toxicity (Figure 3.10) the wastewater line constituents are quite similar in all scenarios, however, the absolute value of the sludge line varies: the larger negative effect to the environment is for MAD, 0.377 kg 1.4-DB eq, and the smaller one is for TPAD, 0.282 kg 1.4-DB eq, which is almost 25% less than that of MAD, and 15% less than TAD. This happens due to the higher total amount of digested sludge produced at mesophilic conditions rather than at TAD or TPAD. In particular for the Human toxicity category, less than 3% of the avoided environmental impacts are given by the energy production at TPAD conditions. The main contributor to this impact category is land application (Sludge disposal_SL) due to the heavy metals and other toxic substances that are still present in the digested sludge (41–45%) – Figure 3.11. The other major contributors are the energy consumption in the wastewater treatment line (26-29%), followed by the water body pollution (Water pollution_WW) made by treated wastewater discharge (19-22%) and, finally, by the different chemicals' consumption (Chemicals_WW) used at different stages of the wastewater treatment processes such as phosphorus precipitation and coagulation (all around 5%).

In terms of the Ionising radiation impact category, even though the absolute values for both lines are lower than ±0.1 kBq U235 eq/m^3 of treated wastewater, the avoided environmental impacts given by the sludge line of WWTP compensates the negative influence of wastewater treatment line for more than 40% for both TAD and MAD, and around 90% for TPAD (Figure 3.10). The latter leads to a better balance of both avoided and overall environmental impacts for TPAD. The rest of the contributions are given by different chemicals' consumption (Chemicals_WW) used for wastewater treatment processes such as phosphorus precipitation and coagulation (all less than 9%) – Figure 3.11. The factors that represent the avoided environmental impact are land application as the final sludge disposal (Sludge disposal_SL) and the energy production (Electricity_SL), both are from the sludge line of WWTP. The percentage contribution of them, respectively, are: 45% and 55% for TAD, 43% and 57% for MAD, 27% and 73% for TPAD. For TPAD, the distribution is significantly different from TAD and MAD due to the better energy balance after AD.

In the context of other impact categories such as Agricultural land occupation, and Metal and Fossil depletion, the avoided impacts of the sludge line made mainly by land application as the final sludge disposal (Sludge disposal_SL) and energy production (Electricity_SL) completely captures the negative influence of wastewater treatment line given by energy consumption (Electricity_WW) and chemicals' consumption (Chemicals_WW) – Figure 3.11.

The environmental impact represented through the rest of the assessed impact categories show relatively low absolute values: <0.0052 kg SO_2 eq/m^3 of treated wastewater for Terrestrial acidification, <0.0011 kg P eq/m^3 of treated wastewater for

Freshwater acidification, and <-1.0*10^{-7} kg CFC-11 eq/m^3 of treated wastewater for Ozone depletion.

Terrestrial acidification impacts are built up due to the gaseous emissions (Air emissions_WW) from the wastewater treatment line, <10% from energy demand (Electricity) and<5% from chemicals (Chemicals_WW) used at wastewater treatment line – Figure 3.11.

Freshwater eutrophication is mostly affected by water body pollution (Water pollution_WW) with a 45-50% contribution – Figure 3.11 – and by energy consumption (Electricity_WW) with a 25% contribution from the wastewater treatment line.

Ozone depletion results are driven by the avoided environmental impacts of both the energy produced (Electricity_SL) – around 60% for TAD and MAD, and more than 65% for TPAD; and the land application (Sludge disposal_SL) - around 20% for TAD and MAD, and around 15% for TPAD – see Figure 3.11. These avoided impacts are significantly bigger than those caused by Electricity_WW and Chemicals_WW consumption.

Concerning the factors mainly contributing to the different environmental impact categories negatively, there are certain ones confirming their prevailing parts in the total environmental burden. In the case of WWTP-LCA, the major contributors are: the gaseous emissions from the open biological step reservoirs to the air, the energy consumption for aeration tanks (Uggetti *et al.*, 2011) and the water body secondary pollution given by treated wastewater discharge – see Figure 3.11. All of them are related to the wastewater treatment line.

For the Climate change impact category, both the gaseous emissions to the air and the energy consumption – again related to the wastewater treatment line – are the biggest contributors to the environmental burden, followed by Chemicals consumption – related to the wastewater treatment line – and the gaseous emissions to air – from the sludge line – with around 10-15% all together.

3.3.2 SL-LCA with mesophilic, thermophilic or temperature-phased anaerobic digestion

The LCA results of the sludge line (SL-LCA) including methane production are presented in Figure 3.12 using the second FU: 1 m^3 of methane produced (unlike Figures 3.10 and 3.11 which use the FU: 1 m^3 of wastewater treated).

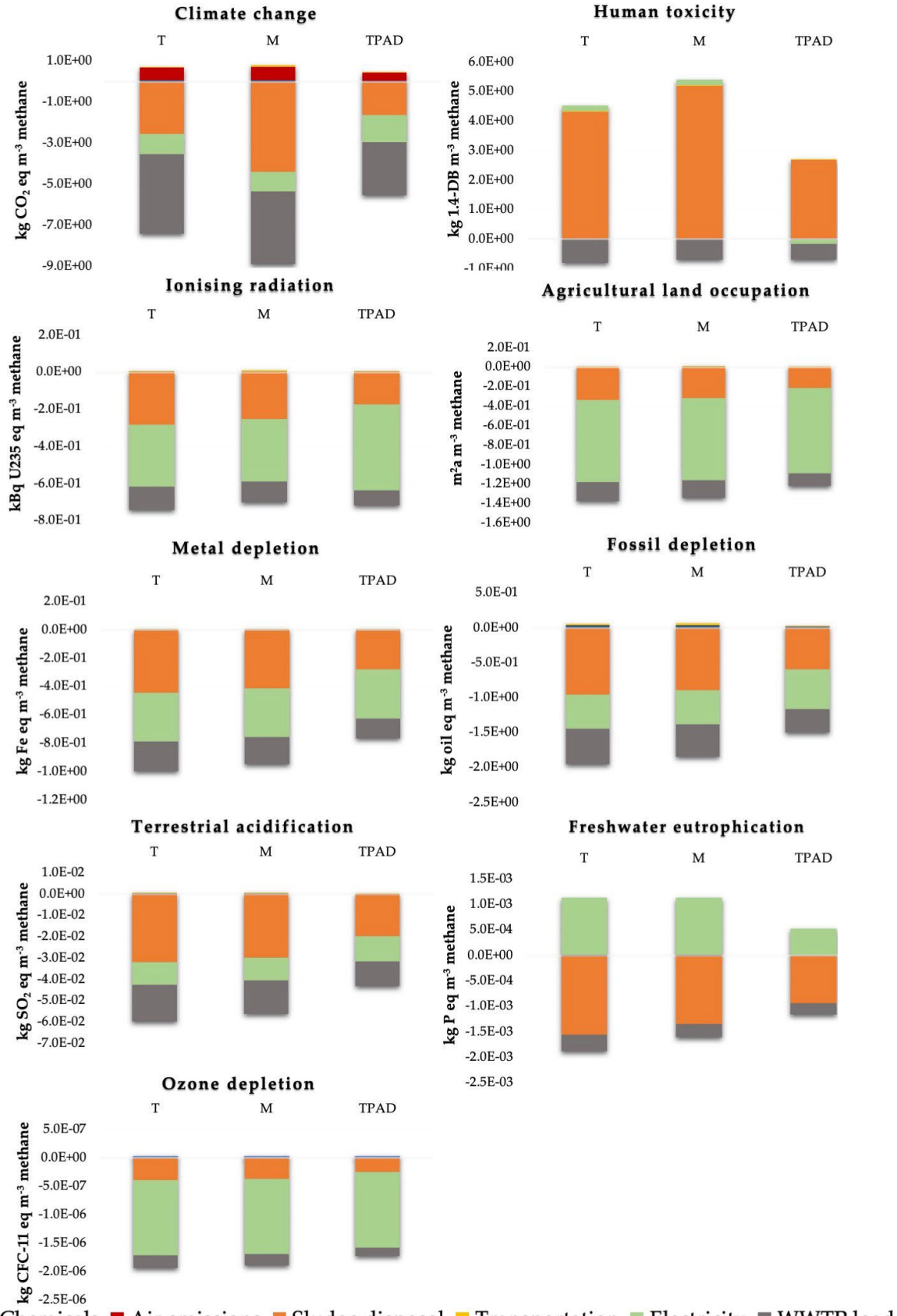


Figure 3.12 – Contribution analysis of the potential environmental impacts for three scenarios of the sludge line (SL-LCA): TAD, MAD, TPAD. Results shown for the FU: 1 m^3 of methane produced

Figure 3.12 includes all environmental impact categories considered in this study, and within each impact category there are three scenarios: mesophilic, thermophilic and TPAD. Furthermore, the different contributions from all process inputs and outputs are included for each of the three scenarios and for all categories. Positive values represent the environmental impacts, while negative values refer to the avoided environmental impacts (here considered as environmental credits).

The aggregated SL-LCA results for the three scenarios lead to overall avoided environmental impacts in all categories with exception of Human toxicity. From the three scenarios, TAD outperforms MAD and TPAD in seven out of the nine impact categories here analysed (except for Climate change and Human toxicity) (Figure 3.12). MAD is consistently the second best scenario in six out of the nine categories, except for Climate change (where it performs the best), and both Ionising radiation and Human health (where it performs worse). Finally, TPAD has the lowest environmental impacts for Human health (by over 50%), while it also has the least avoided environmental impacts for seven categories out of the nine here analysed (Figure 3.12).

In all SL-LCA scenarios, the contributing factors with the largest absolute values (*i.e.* either caused impacts – for Human toxicity – or avoided impacts – for all the other categories) are the final sludge disposal (starting from 15% for Ozone depletion to almost 80% for Human toxicity), energy balance (from 12% for Climate change to over 75% for Ozone depletion) and water pollution (from 11% for Human toxicity and Ionising radiation to 22% for Fossil depletion). It is also important to highlight that the factor of gaseous emissions to the air contributes significantly in a harmful way only for Climate change (more than 80% of caused environmental impact and only less than 8% of total environmental impact) due to the digested sludge accumulated at the landfill (Yu *et al*., 2020).

In the case of specific impact categories, the avoided environmental impacts in Climate change for both TAD and MAD are larger than those of TPAD by 24% and 38%, respectively – Figure 3.12. The only factor causing environmental impacts on Climate change for the three scenarios are the gaseous emissions from AD installations (Air emissions). On the contrary, the avoided environmental impacts have been credited by the following factors: additional reject water treatment (WWTP load concerning each scenario: 53% for TAD, 40% for MAD and 47% for TPAD), final sludge disposal (Sludge disposal: 34% for TAD, 49% for MAD and 29% for TPAD) and energy production (Electricity: 13% for TAD, 11% for MAD and 24% for TPAD). Further minor avoided impacts are related to chemical consumption (Chemicals) (around 10% for all AD types) and transportation (Transportation) (around 5% for TAD and TPAD, and around 11% for MAD due to longer distance – a round trip to the composting site).

In terms of Human toxicity, TPAD demonstrates the best results with the lowest environmental impacts at SL-LCA (Figure 3.12). The TPAD's impacts on Human toxicity are 46% lower than TAD, and 58% lower than MAD. The most substantial contribution to Human toxicity is coming from the final sludge disposal (Sludge disposal – 95% for TAD, 96% for MAD, and 99% for TPAD) which makes sense as it is the agricultural land application for the TAD and TPAD scenarios and agricultural land application via composting for the MAD scenario – Figure 3.12 and Table 2.7. The rest of the impacts on Human toxicity are mostly caused by the energy consumption (Electricity) with 4% and 3%, for TAD and MAD, respectively. While for TPAD, the energy balance is slightly positive, meaning that the system produces surplus energy with respect to its total consumption which leads to avoided impacts by almost 6%. Hence, the TPAD scenario for SL can be considered as energy self-sufficient process and an electricity supplier. Furthermore, for Human toxicity there are some minor avoided impacts from additional reject water treatment (WWTP load – 100% for TAD and MAD and 94% for TPAD) which is highly polluted, meaning that it can be used as an additional source for resource recovery (Khan & Nordberg, 2018; Quist-Jensen *et al*., 2018).

Interestingly, Human toxicity is the only impact category that does not result in overall avoided impacts at the sludge line. This happens due to the sufficient amounts of heavy metals and other toxic pollutants that are not completely removed during AD operation. Knowing that the TAD and TPAD digested sludges are considered to be pathogenically safe, and their final disposal can be a direct land application as fertilizers (Riau *et al.*, 2010). MAD digested sludge undergoes through an additional step of composting prior to its application in agriculture. However, the gaseous emissions as well as the traces of heavy metals (Table 2.7) result in certain danger to the human health (Hao *et al.*, 2019).

Looking at the Freshwater eutrophication impact category, the TPAD scenario shows both the lowest environmental impacts (50% lower than TAD and MAD, with energy consumption – Electricity – as the main contributor) and the lowest avoided environmental impacts. In the latter case, the prevailing contributors are digested sludge usage for the agricultural land application (Sludge disposal) and water body pollution reduction (WWTP load).

The rest of the impact categories (*i.e.* Ionising radiation, Agricultural land occupation, Metal and Fossil depletion, Terrestrial acidification, Freshwater eutrophication and Ozone depletion) follow a similar pattern. For all SL-LCA scenarios, the overall result can be referred as avoided impacts, with the best results being obtained for TAD, followed by MAD, and finally by TPAD. The main contributors are Sludge disposal and Electricity, and the WWTP load to a lower extent (with a maximum of 20% for Terrestrial acidification and lesser for other impact categories).

3.3.3 Sensitivity analysis

The sensitivity response (*i.e.* the sensitivity coefficient "S" as described in the subchapter "**2.3.5 Sensitivity analysis**") of all studied environmental impact categories is analysed with respect to the assumed values for the digested sludge volume (with ±5% of the baseline value for the TPAD scenario, *i.e.* 90332 t/year > 85816 t/year > 81299 t/year). Only the TPAD scenario was considered for sensitivity analysis due to the variability of the experimental data obtained by the previous studies (Riau *et al.*, 2010; Micolucci *et al.*, 2018; Yu *et al.*, 2020) and shortage of the reported data from full-scale WWTPs, especially considering that TPAD is the least spread AD system worldwide among others (Massanet-Nicolau *et al.*, 2015).

The sensitivity coefficients are analysed considering the processing conditions of TPAD for both the WWTP and the SL alone as shown in the Table 3.23.

Table 3.23 – Sensitivity coefficients (S)* and environmental impacts** of the whole WWTP and SL alone with respect to the TPAD baseline value assumed for the digested sludge volume

Case	**WWTP**			**SL**		
Impact category	**S coef-ficient**	**+5%**	**-5%**	**S coef-ficient**	**+5%**	**-5%**
Climate change (kg CO_2 eq/FU)	-0.309	0.543	0.561	0.321	-5.108	-4.938
Human toxicity (kg 1.4-DB eq/FU)	0.431	0.711	0.679	**1.325**	**2.162**	**1.880**
Ionising radiation (kBq U235 eq/FU)	-1.424	0.012	0.014	0.241	-0.722	-0.704
Agricultural land occupation (m^2a/FU)	0.245	-0.096	-0.093	0.173	-1.226	-1.204
Metal depletion (kg Fe eq/FU)	0.530	-0.059	-0.056	0.363	-0.777	-0.748
Fossil depletion (kg oil eq/FU)	0.980	-0.068	-0.061	0.415	-1.495	-1.431
Terrestrial acidification (kg SO_2 eq/FU)	-0.478	$4.41 \cdot 10^{-3}$	$4.64 \cdot 10^{-3}$	0.467	-0.044	-0.042
Freshwater eutrophication (kg 1.4-DB eq/FU)	-0.103	$-9.92 \cdot 10^{-4}$	$-1.0 \cdot 10^{-3}$	**1.504**	$\mathbf{-6.76 \cdot 10^{-4}}$	$\mathbf{-5.77 \cdot 10^{-4}}$
Ozone depletion (kg CFC-11 eq/FU)	0.172	$-1.57 \cdot 10^{-7}$	$-1.54 \cdot 10^{-7}$	0.143	$-1.73 \cdot 10^{-6}$	$-1.71 \cdot 10^{-6}$

Note: * Sensitivity coefficients (S) are unitless;
** Units of each environmental impact category consider the specific FU for WWTP and SL, *i.e.* 1 m^3 of treated wastewater and 1 m^3 of produced methane, respectively.

A positive value of the sensitivity coefficient (S) refers to a straight influence of the studied parameter on the environmental results: *e.g.* the more sludge that is considered, the higher the (avoided) environmental impacts are. On the contrary, a

negative sensitivity coefficient means an opposite influence of the studied parameter on the environmental results, it is: *e.g.* the more sludge that is considered, the less the (avoided) environmental impacts are.

In this study, negative sensitivity coefficients are obtained only for WWTP-LCA, concerning four impact categories: Climate change, Ionising radiation, Terrestrial acidification and Freshwater eutrophication.

In a case of the potential environmental impacts related to the Climate change, Ionising radiation and Terrestrial acidification (Table 3.23), this behaviour occurs due to an increased (proportional to the digested sludge volume) amount of both digested sludge as fertilizer substituent and energy recovered as biogas. In the case of Freshwater eutrophication, this opposite behaviour occurs since an increase in the digested sludge volume leads to an additional amount of highly polluted reject water (that in turn needs to be further treated) generating a minor amount additional environmental impacts but that overall reduces the total avoided impacts.

On the contrary, the sensitivity coefficients for SL-LCA are positive values in all impact categories indicating a positive relation between the input variable (*i.e.* the assumed digested sludge volume) and the out variable (each environmental impact category). In this case, larger digested sludge volumes lead to the larger (either potentially caused or avoided) environmental impacts. In particular for the Human toxicity category, impacts are higher with the increase in the digested sludge volume (due to the proportional increase in the present pollutants in the digested sludge), while all the other categories included in Table 3.23 result in larger avoided environmental impacts with the increment in the digested sludge volume (due to the production of the avoided products such as fertilizers and electricity).

The most sensitive environmental impact categories at SL-LCA in terms of ±5% variation in the digested sludge amount are Human toxicity and Freshwater eutrophication, as S is positive and higher than 1.0.

The digested sludge amount variation of +5% increases the environmental burden of Human toxicity on 6.5% from the baseline value at SL-LCA. At WWTP-LCA, the digested sludge amount of ±5% had the influence of around 2.0% referring to the baseline value. It is also important to mention that the Human toxicity impact category is the only one with positive sensitivity coefficients at both LCAs, and for SL-LCA the sensitivity coefficient is higher than 1.0. Hence, it is important to mention that such sensitivity behavior of the Human toxicity category reveals that the major environmental concern based the variability of the digested sludge amounts would be on this impact category.

For Freshwater eutrophication at SL-LCA, the avoided environmental impacts increase on over 7.0% along with the increment in the digested sludge amount applied in agriculture as a fertilizer. At WWTP-LCA, the sensitivity coefficient at this impact category is negative and lower than 1.0, and can be neglected.

At the WWTP-LCA, the rest of the impact categories (*i.e.* apart from the ones with negative sensitivity coefficient) result on S values lower than 1.0. The sensitivity coefficient values higher than 0.5 are for the impact categories Metal and Fossil depletion. These two impact categories are affected on 2.5% to 5%, respectively (Table 3.23), and they refer to overall avoided environmental impacts. Furthermore, the sensitivity coefficients for Metal and Fossil depletion at WWTP-LCA are higher than those at SL-LCA. A reason for such a difference is the contribution in the energy balance (Electricity_WW) at WW line (Tables 2.6 and 3.23). The absolute values of avoided environmental impacts at ±5% of digested sludge are essentially higher at SL-LCA than at WWTP-LCA for Metal depletion (on over 92%) and for Fossil depletion (on over 95%) due to the energy consumption at the WW line concerning both impact categories.

In general terms, it can be said that the WW line has a higher harmful effect on the environment than SL line itself, and the larger its scale is, the larger the potential environmental impacts will be, contrary to the SL line.

Other general trends from the sensitivity analysis are that the sensitivity gives a clear overview that AD, namely TPAD, affects the environment mainly due to the toxic substances' content and air emissions derived from the digested sludge, which are proportional to its volume. The digested sludge production affects the environment negatively by the contribution to Human toxicity due to the final sludge disposal (Sludge disposal) coming from the SL line which relates to both WWTP-LCA and SL-LCA (Figures 3.10-3.12). At the same time, digested sludge production has also a positive effect given by resource (fertilizer) and energy (electricity and heat) recovery (Sludge disposal and Electricity, respectively) and also due to the additional reject water treatment (WWTP load) derived from the SL line (Figure 3.12).

Therefore, the impact categories of Human toxicity, Metal and Fossil depletion which are directly related to the produced digested sludge amount are of major attention for this type of processes. Considering the case of the TPAD scenario, it can also be said that ±5% of digested sludge production does not affect most of the (avoided) environmental impacts. Only three environmental impact categories have $S > 1.0$, namely: Human toxicity (SL-LCA), Ionising radiation (WWTP-LCA) and Freshwater eutrophication (SL-LCA). These S values grated than unity are strongly related to several contributing factors such as energy consumption (WWTP-LCA), final sludge disposal and reject water treatment (SL-LCA) – Figures 3.10 and 3.11, and Table 3.23.

Hence, these findings of the sensitivity analysis should be considered and taken into account for future designs of WWTPs and AD systems.

Based on the sensitivity analysis performed, it can be said that the main factor that contributes to the environmental impact through Human toxicity impact category is digested sludge quality (pathogenic safety, presence of the toxic substances and gaseous emissions) and its amount. Therefore, by considering and changing the final digested sludge disposal, the total environmental impact can be reduced.

3.3.4 Conclusions

In this study, a comparative LCA analysis was carried out to evaluate the environmental impacts of three alternative AD processes (mesophilic, thermophilic and TPAD) used for sludge treatment in activated sludge WWTP. The environmental burden was evaluated at two scales, namely the whole WWTP (to assess the contribution of the sludge line to the whole WWTP) – with a FU of 1 m^3 treated wastewater – and the sludge line alone (to highlight the potential environmental benefits from methane production as an additional function of the system beyond the wastewater treatment) – with a FU of 1 m^3 produced methane.

In the WWTP-LCA, five (Climate change, Human toxicity, Ionising radiation, Terrestrial acidification and Freshwater eutrophication) out of the nine environmental impact categories analysed showed potential environmental impacts. The rest of the environmental impact categories (Agricultural land occupation, Metal and Fossil depletion, Ozone depletion) showed avoided environmental impacts, since the WW line led to potential environmental impacts in all impact categories, while the SL line led to avoided environmental impacts for most environmental impact categories (except for Human toxicity). Among all scenarios, the WWTP with TPAD outperformed those with mesophilic and thermophilic AD in all the environmental impact categories, besides Climate change.

The SL-LCA showed mostly avoided impacts, being the highest for thermophilic AD, followed by mesophilic AD and TPAD, except for Climate change where mesophilic AD was the most beneficial. The only potential environmental impact was Human toxicity, being the lowest for TPAD. Differences between both LCA results should be attributed to the FU.

It can be stated that TPAD implemented at activated sludge WWTP (WWTP-LCA) is the most environmentally beneficial among other AD systems investigated in this study.

Within only the SL line, TAD is the most environmentally friendly option, even though its superiority is less evident than of TPAD at WWTP-LCA.

However, as from the technological point of view, any AD of WAS is normally considered within WWTP where it is incorporated, it can be concluded that environmentally-wise TPAD is the most grounded AD systems among all three discussed.

In addition, it can be also concluded that such products as nutrients and energy recovered from the AD systems and incorporated into the sludge treatment create an amount of credits that make the whole WWTP more environmentally friendly.

Chapter 4 – Conclusions and future perspectives

4.1 Conlcusions

In this study, there was investigated the AD process going on in three different combinations of temperature and configuration regimes in the laboratory conditions: thermophilic and mesophilic single-stage AD systems and double-stage temperature-phased AD.

There were two technological aspects considered in addition to the evaluation of standard efficiency parameters such as organic matter degradation efficiency and methane production: mixing efficiency through the stirrer construction and velocity regime and quality of the digested sludge, namely its dewaterability. Both these AD aspects are of crucial engineering and economic interest at full scale WWTPs which became a reason of thorough research.

In this study, two different types of mixing mechanisms (a simple flat one-straight-blade paddle agitator located at the bottom of the reactor performed and a more complicated two-straight-blade paddle impeller system) with two rotational regimes (95 rpm for the fermenter and 100 rpm for other three reactors and 30 rpm for the fermenter and 50 rpm for others) were constructed and tested. The experiments showed that the more simple mixing mechanism at slower mixing velocity affects AD efficiency in a better way than the more complicated mixing system at higher rotational speed, having as an aim the highest methane production.

In addition, for further research the discontinuous mixing with short and long intervals could be tested to come up with the most efficient solution in AD mixing.
The quality of the digested sludge was another critical issue in this study.

The dewaterability was in the center of the focus as the most economically wise crucial and, apparently, the most complex parameter of the digested sludge which forms a core around the dewatering process and its efficiency.

Two methods of mechanical dewatering (which is normally used at the full-scale WWTPs and the results of which can be related to the laboratory results) were applied to test all three types of digested sludges formed in the laboratory conditions.

The mechanical pressing results turned out to be insignificant statistically. Nevertheless, the polymer consumption was the highest for TAD sludge. Oppositely, after centrifugation, the best dewaterability properties belonged to the digested sludge from TAD and TPAD. Given the highest polymer consumption for TAD, in terms of such a complex property of digested sludge as dewaterability, TPAD as a combination of thermophilic and mesophilic conditions represents the best outcome.

Another meaningful finding was that TPAD sludge was similar in pathogenic safety to TAD sludge. Given that the HRT of TPAD1 was only 2 days applied for all four experimental Phases, the obtained results look promising concerning the opportunity to use digested sludge as a fertilizer in agriculture.

Based on the lower calorific value, the higher energetic value increases its efficiency when incinerated which suits at most to the digested sludge after MAD. The lower energetic value goes alongside with the higher pathogenic safety, which allows to use such digested sludge for agricultural application. However, when the lowest energetic value belongs to the digested after TPAD, the highest pathogen deactivation was achieved by TAD.

To summarize, it can be stated that the experimental results showed that the TPAD was the most beneficial in terms of organic matter degradation efficiency (32.4% against of 27.2 for TAD and 26.0 for MAD), producing a digestate with high dewaterability (on 8.1-9.8% worse than for TAD and on 6.2-12.0% better than for MAD) and pathogenic safety (coliforms and *Escherichia coli* were not detected, and *Clostridium perfringens* were counted up to 4.8-4.9 $\times 10^3$, when for TAD it was only 1.4-2.5$\times 10^3$, and for MAD it was 1.3-1.8x10^4), with the lowest LCV (19.2% against 15.4% and 15.8% under thermophilic and mesophilic conditions, respectively). Regarding the final disposal, the digested sludge after TAD can be applied directly in agriculture, after TPAD, it can be used as a fertilizer only in case the fermenter HRT assures the pathogenic safety. The MAD digestate is the best for being used as a fuel preserving higher portion of organic matter, not transformed into biogas during AD.

There were also performed two LCAs with two different FUs for the overall environmental assessment of the three independent AD systems. LCA as an analytical tool allowed to reveal the main bottlenecks of each AD system itself and also within the classic WWTP. The TPAD system implemented at activated sludge WWTP (WWTP-LCA) is the most environmentally beneficial among other AD systems investigated in this study. Meanwhile, within only the SL line, TAD is the most environmentally friendly option, even though its superiority is less evident than of TPAD at WWTP-LCA.

4.2 Future research

As a further step of the investigation, it can be suggested to conduct other methods of mechanical dewatering or performing then at pilot-scale to be able to obtain the comparable results to those got from the full-scale. The polymer agent dose can be also tested for each type of the sludge when produced at the pilot AD plant.

As another objective for further research, in addition to both AD aspects studied and LCA application, the economic evaluation could be useful to support the technological solutions and environmental perspectives.

Appendix A

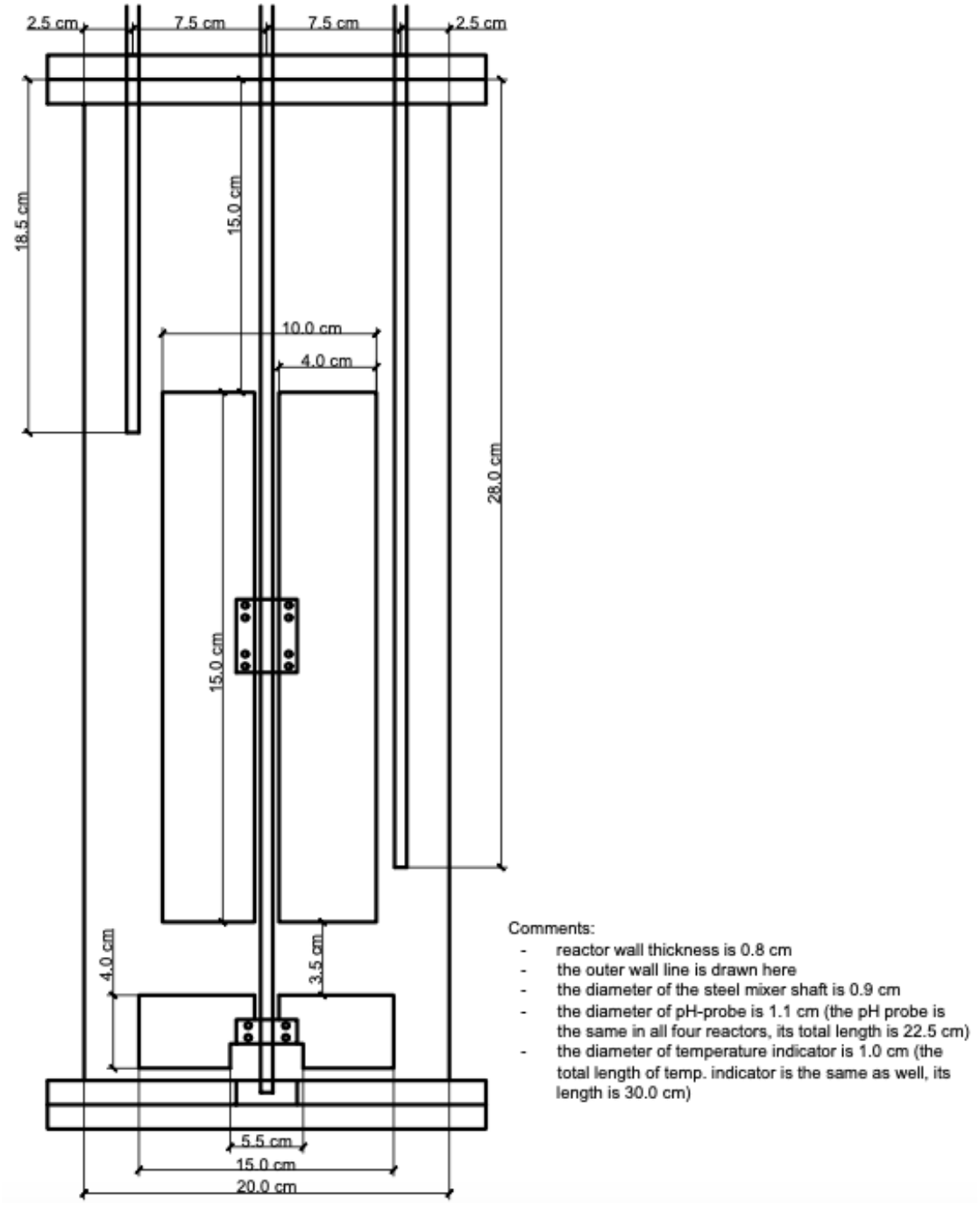


Figure A.1.1 – A technical drawing of a big reactor (T, M, TPAD2) with an additional vertical paddle (the drawing corresponds to the Phases B and D)

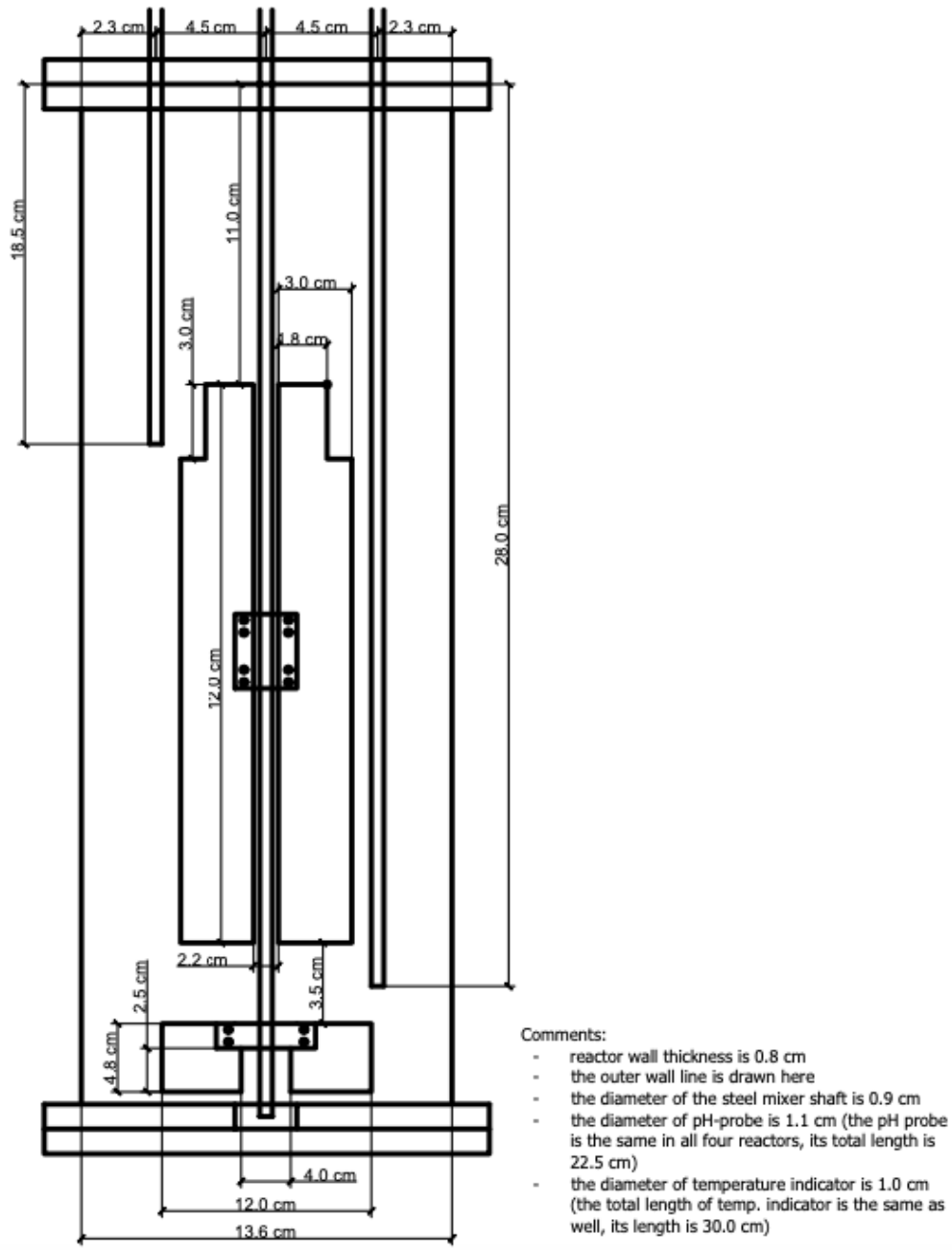


Figure A.1.2 – A technical drawing of a small reactor (TPAD1, or fermenter) with an additional vertical paddle (the drawing corresponds to the Phase B)

Appendix B

Table B.1.1 – The abundance of microorganisms in the laboratory reactors with distinction to the moderate (+), considerable (++) and high (+++) abundance.

date of	Reactor type	MAD			TAD			TPAD1			TPAD2		
samp-ling	Level**/ FISH probe	High	Med.	Low	High	Med.	Low	High	Med.	Low	High	Med.	Low
17.10. 2018	EUBmix	+				+							
	ARCH915	++	++	++	+	+	+	++	+	+	+	+	+
19.12. 2018	EUBmix		+										
	ARCH915	+++	++	++/+++	+	+	+	+	+	+	+	+	+
13.05. 2019	EUBmix	+	++										
	ARCH915	+	+	+	sc*		sc*	+	++	sc*	+	+	++
10.08. 2019	EUBmix	+						-	-	-			
	ARCH915	+	+	+	sc*	sc*	sc*	-	-	-	+	++	+

Table B.1.2 – The methanogenic consortia presented in TPAD1 with distinction to the considerable (++) and high (+++) abundance as well as single colonies (sc)

date of sampling	reactor:	TPAD1		
	level/FISH probe	D1	D2	D3
10.08.2019	EUBmix			
	ARCH915	++	++/+++	sc*

Note to the tables C1.2.2 and C1.2.3:
*sc – single colonies;
- – absence of a sample;
empty cell – no colonies;
Legend:
**Level
Low – the level of the lowest reactor valve;
Med. – the level of the middle reactor valve;
High – the level of the highest reactor valve

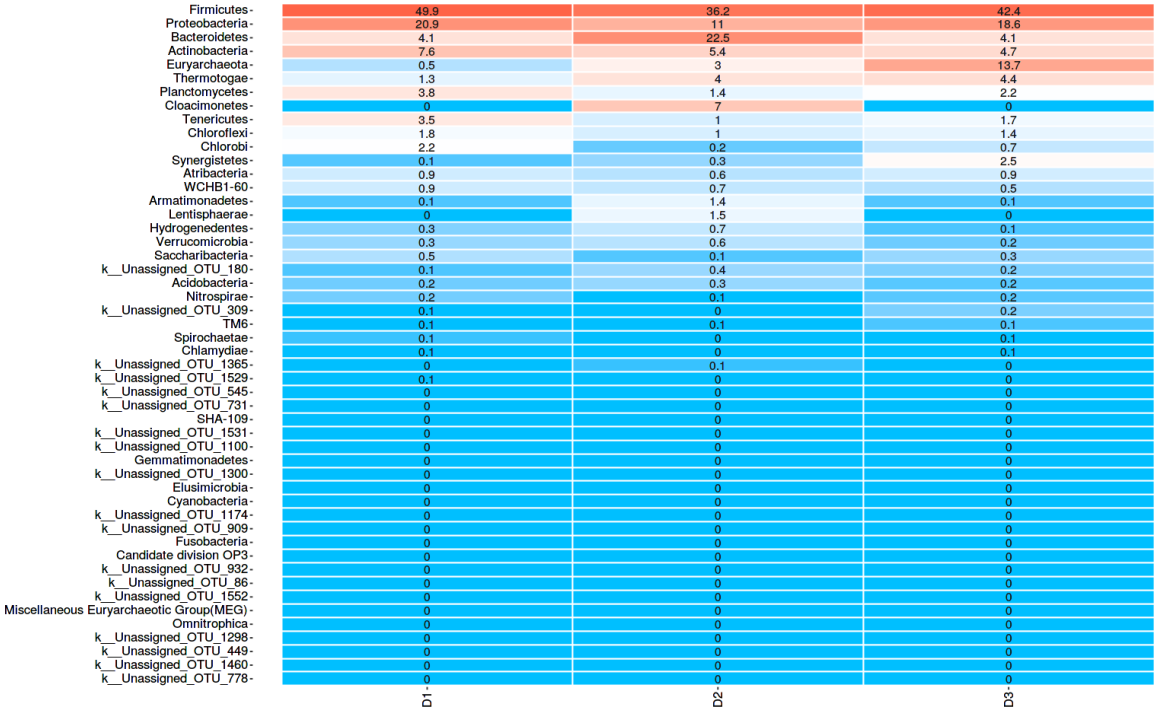


Figure B.1.1 – The 50 most abundant phyla across all samples

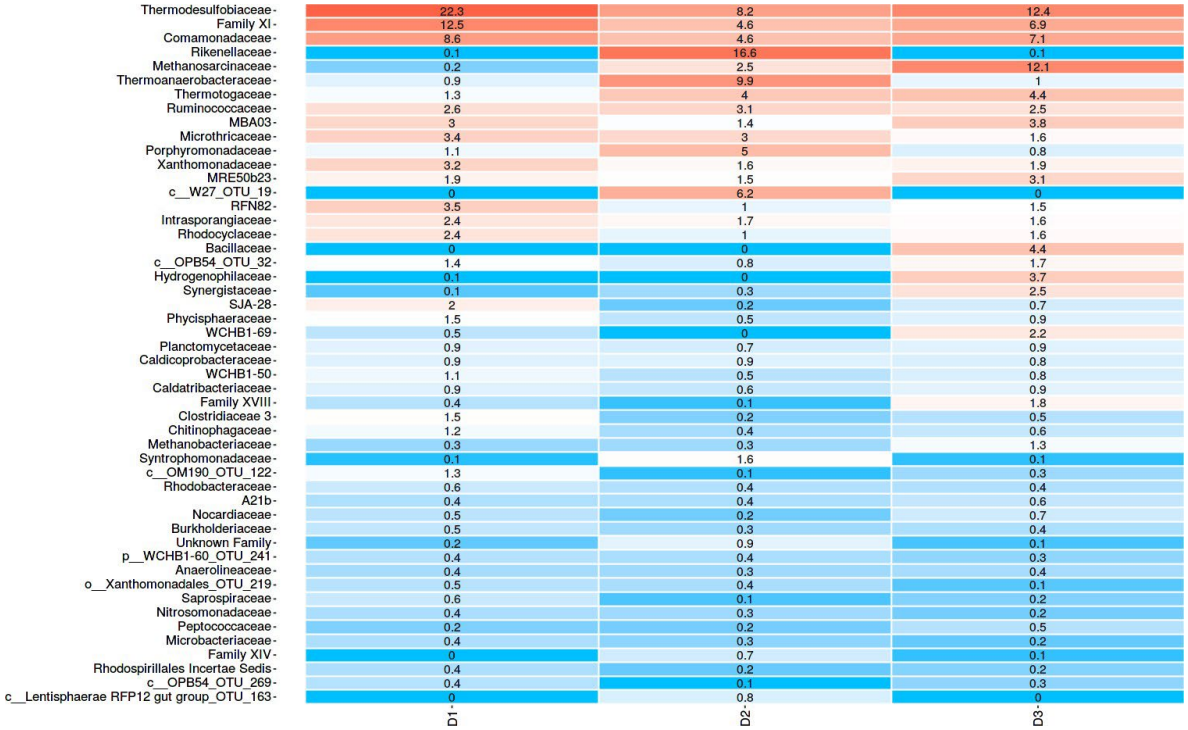


Figure B.1.2. – The 50 most abundant families across all samples

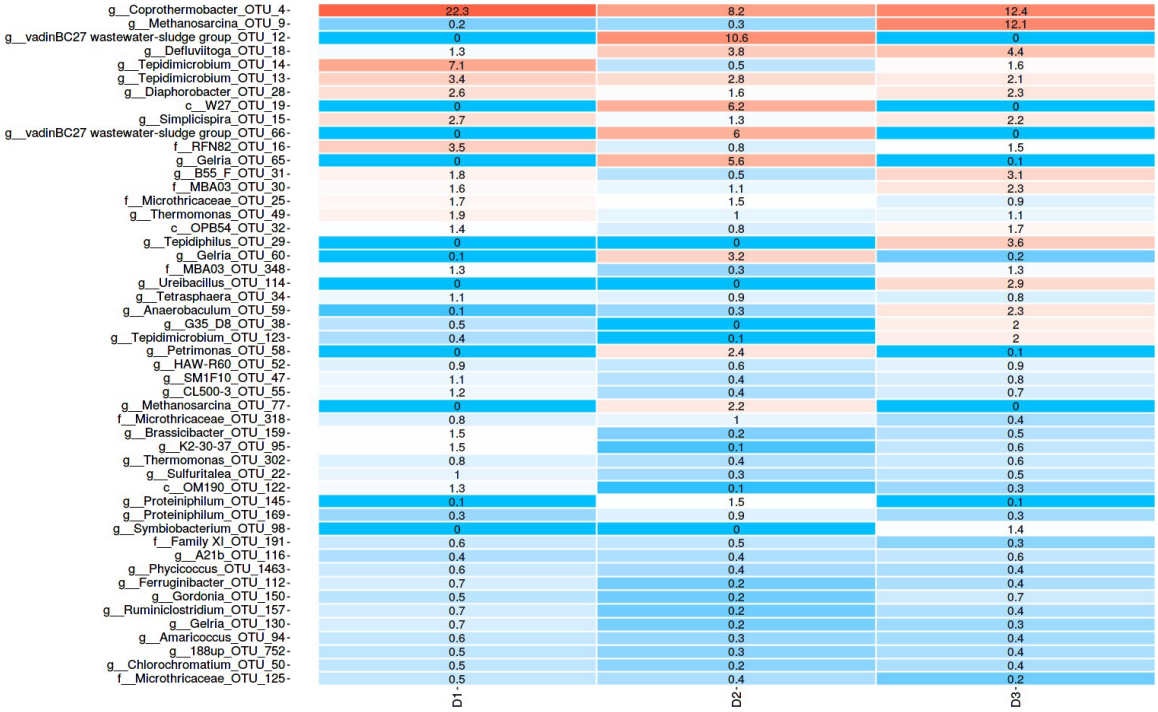


Figure B.1.3 – The 50 most abundant species across all samples

Chapter 5 – Literature

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Chapter 6 – List of publications

Published paper:

Lanko, I., Flores, L., Garfí, M., Todt, V., Posada, J.A., Jenicek, P. and Ferrer, I. 2020. Life Cycle Assessment of the Mesophilic, Thermophilic, and Temperature-Phased Anaerobic Digestion of Sewage Sludge. *Water.* Vol.12 (11), 31-40. https://doi.org/10.3390/w12113140

Re-submitted paper:

Lanko, I., Hejnic, J., Říhová Ambrožová, J., Ferrer, I., Jenicek, P.
Digested Sludge Quality in Mesophilic, Thermophilic and Temperature-Phased Anaerobic Digestion Systems.

Paper in preparation:

Lanko, I., Milobedzka, A., Šoóš, M., Hejnic, J., Kurpas, J., Bartackova, J., Ferrer, I., Jenicek, P.
Mixing efficiency in three types of AD systems: thermophilic, mesophilic and temperature-phased.