Viljami Kinnunen

Anaerobic digestion of microalgae and pulp and paper biosludge

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Abstract

In recent decades, microalgae have attracted attention as a promising biomass source for a variety of different biofuels, including methane via anaerobic digestion (AD). However, the energy intensity and cost (e.g., for the nutrient supply) of the process chain mean that breakthroughs in algal biofuels have yet to be realized. The objective of this study was to improve the AD of wastewater-grown microalgal biomass, marine algal residues following lipid extraction for renewable diesel production and to improve the AD of pulp and paper industry biosludge. The digestate from the latter substrate could provide nutrients for algae cultivation and lipid extraction followed by AD offers the possibility of obtaining multiple products from algal biomass, as envisaged by the algal biorefinery concept.

Based on the results of this experimental work, pretreatments and novel reactor designs can be used to improve the AD of microalgae. In this study, BMPs for wastewater- and digestate-grown mixed populations of microalgae varied between 154 and 273 L CH_4 kg^{-1} volatile solids (VS). Low-temperature (3 h, 80°C) pretreatments enhanced the BMPs by 11–27%. However, to ensure positive energy balances, the availability of waste heat was necessary. Due to longer solid retention times, the AD of microalgae in unmixed, accumulating-volume reactors (AVRs) at 16–21° C was more feasible than AD in conventional completely stirred tank reactors (CSTRs) at 35°C when the solid concentration of the algal biomass was low (< 4% total solids [TSs]). Biological (at ~60°C) and freeze-thaw pretreatments enhanced the methane yield (32–50% increase) and the mineralization of nitrogen and phosphorus (41–84% increase) in the low-temperature AVRs.

In the present study, the AD of marine algae residue after lipids were extracted for renewable diesel production was demonstrated and the salt concentration of the marine algal biomass did not affect AD. Thermophilic AD in the CSTR resulted in a 48% higher methane yield (220 L CH_4 kg^{-1} VSs) of algal residues compared with mesophilic AD. However, unlike mesophilic AD, ammonia, which originated from the high nitrogen content of the algal biomass, inhibited the thermophilic process.

AD of pulp and paper industry biosludge mineralized nutrients to a soluble form, making effluent a potential media for algal cultivation. The methane yield from the biosludge was low (78 L CH_4 kg^{-1} VS) but increased by 77% with thermal pretreatment (20 min, 121°C). The pretreatment also resulted in AD with a retention time of 10 d, as compared to 14 d for untreated biosludge. However, the energy balance of the pretreatment was dependent on the solid concentration and temperature of the biosludge from the industrial process.

To conclude, this work demonstrated AD of microalgae under psychrophilic, mesophilic, and thermophilic conditions. The low energy balances emphasize that improvements in algae cultivation are required and/or other benefits (e.g., nutrient recovery, value-added products, and waste treatment) obtained for algal AD to become a full-scale application.

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Preface

The experimental work for this thesis was carried out at Tampere University of Technology (TUT), Finland; National Institute of Water and Atmospheric research (NIWA), New Zealand and University of Jyväskylä (JYU), Finland. The research was funded by Maj and Tor Nessling Foundation during the years 2011–2014. The last part of this thesis was written with funding from the TUT graduate school. I wish to thank Maj and Tor Nessling foundation and TUT graduate school for the funding enabling this thesis.

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Viljami Kinnunen

Contents

List of Symbols and Abbreviations

AD	anaerobic digestion
AVR	accumulating volume reactor
COD	chemical oxygen demand
CSTR	completely stirred tank reactor
HRAP	high rate algae pond
HRT	hydraulic retention time
OLR	organic loading rate
sCOD	soluble chemical oxygen demand
S_D	solubilization degree (COD solubilization)
SRT	solid retention time
VFA	volatile fatty acids
VS	volatile solids
VS	volatile suspended solids
TKN	total kjeldahl nitrogen
TS	total solids
TSS	total suspended solids
TVFA	total volatile fatty acids

List of Publications

This thesis consists of following original research papers and manuscripts, which are referred by the Roman numeral in the text

I.   Kinnunen, V., Koskinen, P., Rintala, J. Mesophilic and thermophilic anaerobic laboratory-scale digestion of Nannochloropsis microalga residues. Bioresource Technology 155: 314–322.

II.   Kinnunen, V., Craggs, R., Rintala, J. Influence of temperature and pretreatments on the anaerobic digestion of wastewater grown microalgae in a laboratory-scale accumulating-volume reactor. Water Research 57: 247-257.

III. Kinnunen, V., Ylä-Outinen, A., Rintala, J. Mesophilic anaerobic digestion of pulp and paper industry biosludge–long-term reactor performance and effects of thermal pretreatment. Water Research, 87: 105–111.

IV.   Kinnunen, V., Rintala, J. The effect of low temperature pretreatment on solubilisation and biomethane potential of Chlorella vulgaris and native boreal microalgae biomass grown in synthetic and waste originated media. Bioresource Technology, 221: 78–84.

Author's contribution

I.   I planned the experiments together with Dr. Koskinen and Prof. Rintala, and I conducted most of the experimental work as well as writing the first manuscript draft, which was finalized with all co-authors.

II.   I planned the experiments together with Dr. Craggs, and I conducted the experimental work as well as writing the first manuscript draft with assistance of Prof. Rintala. The manuscript was finalized with all co-authors.

III. I planned the experiments together with Prof. Rintala and conducted the experimental work together with M. Sc (tech.) Ylä-Outinen. I wrote the first manuscript draft, which was finalized with all co-authors.

IV.   I planned the experiments together with Prof. Rintala and conducted the experimental work. I wrote the first manuscript draft, which was finalized together with Prof. Rintala.

1 Introduction

In the past few decades, a variety of biomass sources have been explored for the production of sustainable energy carriers to replace fossil fuels. However, a closer look reveals that many of these alternatives have serious drawbacks. For example, energy crops may compete with food production (Ho et al. 2014), and the mass production of oil plants has led to a vast conversion of forests and peatlands to plantations (Hansen et al. 2014). These changes in land use can have a serious impact on biodiversity and hinder the sustainability of produced fuels from the life cycle perspective (Immerzeel et al. 2014, Hansen et al. 2014, Uusitalo et al. 2014). Microalgae had already been studied in the 1950s as a potential food source for the growing population (Spolaore et al. 2006); at around the same time, they were also investigated to treat wastewater, and algal biomass was suggested for the first time as a possibility for methane production via anaerobic digestion (AD) (Golueke et al. 1957). Unfortunately, despite some promising results, interest in microalgal biofuels faded, and they never became a full-scale technology. During the last decade, interest in producing microalgal biomass for biofuels has been revived, generating intensive research efforts (e.g., reviews by Passos et al. 2014a, Chaudry et al. 2015, Chen et al. 2015).

The main reason for the continued interest in microalgae for renewable energy production is their ability to produce biomass much faster than plants without competing with food production or requiring land use changes (Chaudry et al. 2015, Chen et al. 2015). Furthermore, microalgae can be harvested continuously and use CO_2 for their growth, offering a method to capture carbon from flue gases (Chen et al. 2015). Another advantage is the versatility of microalgae; several alternative types of biofuels can be produced from algal biomass, such as biodiesel, biomethane, bio-syngas, biohydrogen, bioethanol, and biobutanol (Li et al. 2008, Barros et al. 2015). Besides the potential for biofuel production, microalgae may contain several compounds valuable for the chemical industry (e.g., pharmaceuticals and cosmetics) (Mata et al. 2010, Barros et al. 2015).

Despite the high potential of and the intensive research into microalgal biofuels, they have remained nonviable. Several factors cast doubt not only on the viability of a biofuel economy but on biofuel sustainability as well. In fact, the energy balance of the entire algae biofuel system may turn negative (Sills et al. 2012, Kouhia et al. 2015a, Bravo-Fritz et al. 2016, Pragya and Pandey 2016). In particular, the energy-efficient cultivation of desired microalgal species and the harvesting of algal biomass from the cultivation media (mostly water) have been shown to be challenging steps in the process (Barros et al. 2015, Chen et al. 2015). Microalgae can be cultivated in open ponds or closed photobioreactors, with the latter providing better control and biomass production at the cost of higher energy consumption. The use of chemical fertilizers for cultivation is another important factor that decreases the sustainability of algal biofuels (Lam and Lee 2012, Barros et al. 2015). The challenges in both algae biomass production and the energy conversion pathways hinder the energy balance of algal biofuels. For instance, before lipids are extracted from microalgae for biodiesel production, the algal biomass requires energy consuming dewatering or complete drying (Sills et al. 2012, Collet et al. 2014). As a result, it has been suggested that direct methane production through AD could be a better method of energy generation compared to biodiesel production, because it requires no drying

of the algal biomass (Sialve et al. 2009). However, the degradability of microalgal biomass in AD is limited, and methane yields are relatively low. Due to these reasons, the overall energy conversion efficiencies of microalgae to desired fuels have been low (Lam and Lee 2012, Barros et al. 2015).

One solution to make microalgal biofuels a feasible option is to decrease the input resources to the system. Instead of conventional fossil fertilizers, the use of waste nutrients (e.g., wastewater) has been considered vital for algal biofuels (Barros et al. 2015). Microalgae can grow efficiently in wastewater in relatively simple high-rate algal ponds (HRAPs) (Craggs et al. 2012, Chen et al. 2015). In addition, the liquid fraction of digestate from the AD process has been successfully applied for microalgae cultivation (Fouilland et al. 2014, Hidaka et al. 2014, Uggetti et al. 2014, Morales-Amaral et al. 2015). Other wastes or by-products may also have potential; for instance, wastewater and digested biosludge from the treatment of pulp and paper mill wastewater have been recently suggested for use in algae cultivation after AD (Kouhia et al. 2015b, Wieczorek et al. 2015). At present, biosludge is merely a waste material, and it is one with substantial disposal costs. By the AD of biosludge, methane is produced, the nutrients bound to biomass are released to soluble form, and the effluent from the digestion process is available for microalgae production (Kouhia et al. 2015b, Polishchuk et al. 2015).

The ability of microalgae to produce biomass faster than multicellular plants is seriously hindered by the low concentration of the biomass in the growth medium, which makes harvesting challenging. Consequently, algae harvesting is often among the most energy-demanding steps of algal biofuels production. It has even been stated that no efficient and economically viable harvesting method exists at the moment (Barros et al. 2015). Simple gravity settling and the possibility of using algal biomass without a further concentration process (such as drying) could provide substantial energy savings. The challenge in settling is that it may be a slow process and that the highest solid concentrations achieved so far have been around 3% total solids (TS) (Barros et al. 2015); this is still a dilute substrate even for AD, which is usually applied with solid concentrations around 3–15% in wet processes.

In addition to decreasing input resources, another approach to improve the feasibility of algal biofuels is to increase the product output. In the case of AD, microalgae degradability and methane yield can be improved by pretreatments (Ometto et al. 2014, Passos et al. 2014a). However, microalgal biofuels are generally low-cost bulk products, which is still far from economic feasibility; the production of different high-value products, such as astaxanthin, β-carotene, docosahexaenoic acid (DHA), and eicosapentaenoic acid (EPA) from algae and the application of the residue for energy production could be feasible in the short term (Polishchuck et al. 2015, Suganya et al. 2016). At present, high-value products are mainly nutraceuticals, which could limit the use of wastewater or effluent as growth media due to hygienic issues.

The concept of the algal biorefinery is intended to combine the production of high-value compounds and biofuels or energy from algal biomass (Soh et al. 2014). The concept may also benefit from the integration of other factors, such as using wastewater as a nutrient source for algae and exploiting surplus heat from industrial processes. As an example, Kouhia et al. (2015b) presented a microalgae biorefinery where pulp and paper industry wastewater sludge

was used for algae cultivation after AD. From the algae biomass, ω–3 fatty acids, methane, and fertilizer are produced, and the residual algae biomass is directed to AD, producing methane.

The objective of the present thesis was to study AD to produce methane from algal residues after lipid extraction for diesel production, wastewater- or digestate-grown microalgae, and pulp and paper biosludge, aiming to improve methane yield with pretreatments and reducing energy input with a low-cost anaerobic digester design. Figure 1 summarizes the core research areas of this thesis. In this thesis, a literature review on AD of microalgae and pulp and paper biosludge is first provided, following the methods and results and discussion of the experiments. At the end of the thesis conclusions and recommendations for further research are given.
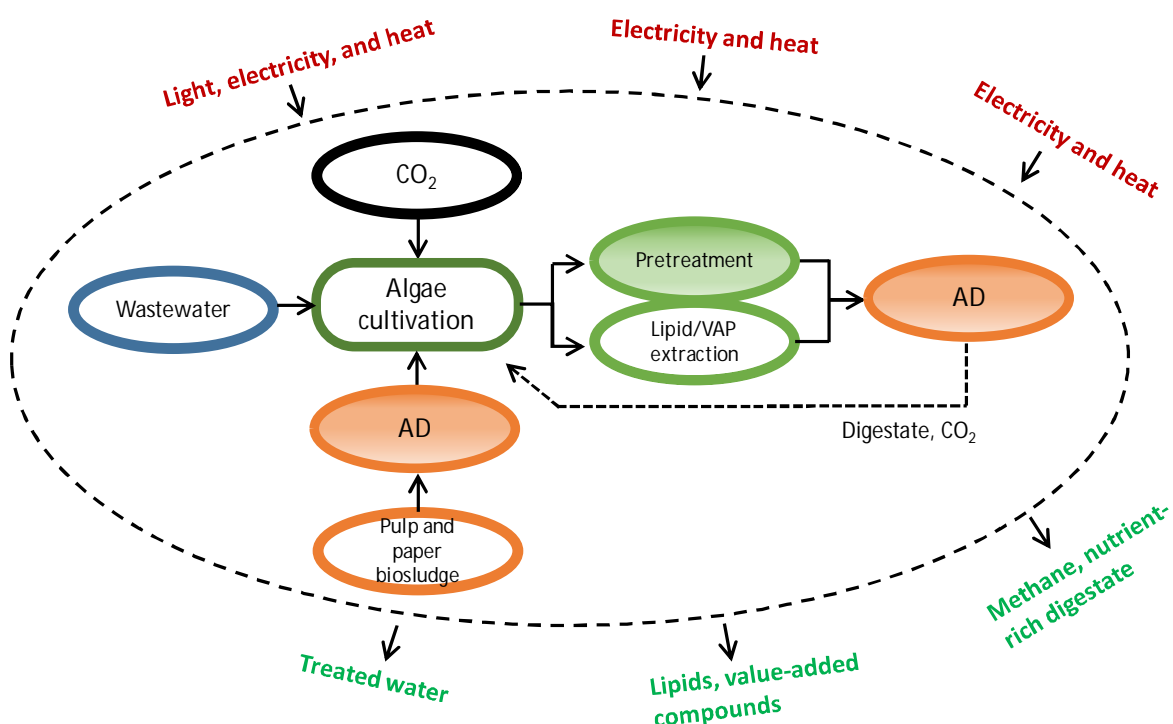

Figure 1. An example of the algal biorefinery concept. The filled shapes present the core research areas presented in this work.

2   Microalgae

Microalgae include both eukaryotic and prokaryotic microorganisms with unicellular or simple multicellular structures. Common to all microalgae is their ability for photosynthesis, as they contain chlorophyll a (Tomaselli 2004). Eukaryotic microalgae may contain green algae (Chlorophyta) and diatoms (Bacillariophyta), while the most common example from prokaryotic microalgae is cyanobacteria (Cyanophyceae) (Li et al. 2008). As microalgae have spread all over the world, there exists a great variety of species. Approximately 40,000 species have been identified (Elliott et al. 2012). Microalgae have adopted different types of metabolisms, and some species can shift from one metabolism to another as a response to changes in environmental conditions. Microalgae can grow photoautotrophically (with light as the energy source and CO_2 as the carbon source), heterotrophically (with organic compounds as the source of both energy and carbon), mixotrophically (with light or organic compounds as the energy source and both organic compounds and CO_2 as carbon sources) and photoheterotrophically (requiring light as an energy source to use organic compounds as a carbon source) (Chojnacka and Marquez-Rocha 2004).

Photosynthetic microalgae need water, light, a carbon source, nutrients, and inorganic salts for their growth. Approximately 50% of microalgal dry biomass is carbon (Chen et al. 2015). Microalgae can produce biomass with growth rates that are a factor of 50 higher than that of the fastest growing terrestrial plants (Li et al. 2008), doubling their biomass even in 3.5 h and commonly within 24 h (Chisti 2007). In addition to high biomass production, microalgae may contain significant amounts of intracellular lipids, which are suitable oils for biodiesel or renewable diesel production. Lipid content varies greatly depending on growth conditions and the algal species (Spolaore et al. 2006, Mata et al. 2010). In normal growth conditions, microalgae synthesize glycerol-based membrane lipids, mainly glycerolipids and phospholipids. Glycerolipids consist of fatty acids with a C10–C20 carbon structure (Hu et al. 2008). These lipids are functional components existing in membrane structures. Under stress conditions, many microalgae species start to synthesize nonpolar (neutral) storage lipids, mostly triacylglycerol (TAG). TAGs are usually located in the cell cytoplasm and work as storage of carbon and energy (Hu et al. 2008). The biomass production potentials for microalgae and some terrestrial plants used currently for biofuel production are shown in Table 1.

Table 1. Biomass production potential (yields) of microalgae and terrestrial plants used for biofuel production. Adapted from Nascimento et al. (2014)

	Biomass yield (tons TS ha^{-1} a^{-1})	Oil yield (m^3 ha^{-1} a^{-1})	Primary energy via AD (MWh ha^{-1} a^{-1})
Microalgae, HRAP			
High productivity	37–82	6–23	83–185[e]
Low productivity	16–36	3–15	36–81[e]
Microalgae, photobioreactors			
High productivity	78–158	32–44	176–356[e]
Low productivity	32–69	7–28	72–155[e]
Energy crops (e.g., for ethanol)			
Maize[a]	11–30	n.a.	33–89
Grass[a]	6–13	n.a.	17–36
Sugarcane[b]	21	n.a.	n.a.
Corn[b]	10	n.a.	n.a.
Oil plants (e.g., for diesel)			
Palm[c]	n.a.	4–6[c]	n.a.
Rapeseed[d]	14–18	1.0–1.2	n.a.

[a] Seppälä (2013), calculated using: grass VS/TS 85%, 330 L CH$_4$ kg^{-1} VS, maize /TS 95%, 350 L CH$_4$ kg^{-1} VS
[b] Somerville et al. (2010), [c] Ong et al. (2011) [d] Budzyński et al. (2015) [e] Calculated using algae VS/TS 90%, 250 L CH$_4$ kg^{-1} VS; n.a. = not applicable

2.1 Cultivation and harvesting of microalgae

Microalgae can be cultivated using indoor or outdoor systems, with the former utilizing sunlight and the latter artificial lighting. However, the need to apply artificial light may cause the energy consumption of algae cultivation to increase substantially even with low-consumption light-emitting diode technology (Kouhia et al. 2015b). On the other hand, the availability of sunlight is dependent on geography. Furthermore, cultivation infrastructure can be divided into open systems, such as HRAPs (Figure 2a), and closed photobioreactors (Figure 2b and 2c). Closed systems enable better control over cultivation parameters and the cultivation of single cultures of algae. In closed systems, the risk of contamination (e.g., by some unwanted algae species or predator zooplankton that could feed on algae) is reduced (Mata et al. 2010). However, the investment and operating costs as well as the energy consumption of photobioreactors are usually considered higher compared to open ponds (Bravo-Fritz et al. 2016).
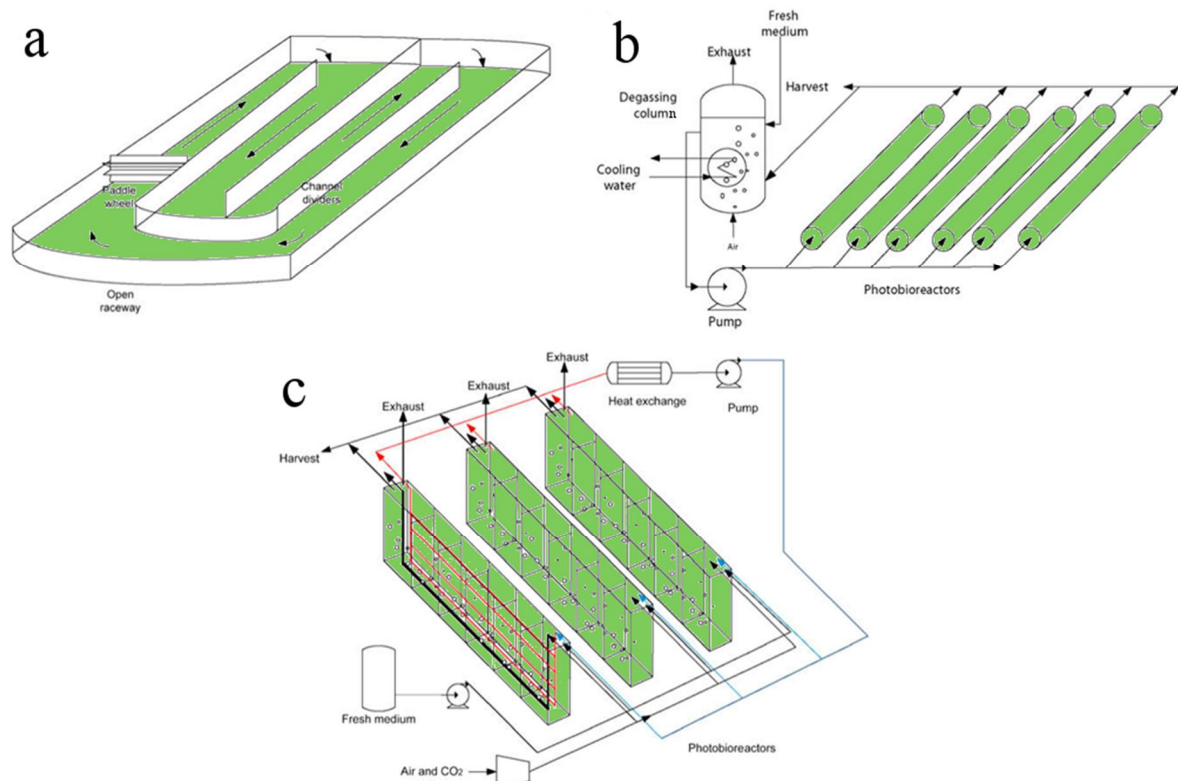
Figure 2. Schematic illustration of microalgae cultivation systems; **a:** Open raceway pond (e.g., high-rate algal pond (HRAP)); **b:** tubular photobioreactor; and **c:** flat-plate photobioreactor. Adapted from Jorquera et al. (2010).

Microalgae require substantial amounts of nutrients and organic or inorganic carbon for their growth. To produce 100 t of microalgae biomass, about 200 t of CO_2, 5 t nitrogen, and 1 t of phosphorus is needed (Morales-Amaral et al. 2015). Although photosynthetic microalgae can uptake atmospheric CO_2, due to its low concentration in the air, additional dosing of CO_2 is needed (Chiu et al. 2009). Biomass production may decrease up to 80% without an external carbon source (Rezvani et al. 2016). Utilization of CO_2 from flue gases (e.g., from power plants burning fossil fuels) and biogas has been demonstrated, although impurities may cause challenges (Zhao and Su 2014).

Microalgae uptake nitrogen from wastewater primarily in the form of ammonium (NH_4^+) and secondarily in the form of nitrate (NO_3^-), while phosphorus uptake occurs in the form of phosphates ($H_2PO_4^2$, HPO_4^-) (Wang et al. 2008). Several studies have shown that the use of synthetic fertilizer has a major environmental impact on microalgae cultivation and may turn the life cycle energy balance negative, as the production of ammonia fertilizers in particular is an energy-intensive process (Wu et al. 2014, Morales-Amaral et al. 2015, Pragya and Pandey 2016). As a consequence, to improve the sustainability of microalgal biofuels during the whole life cycle, it is proposed that the use of waste-originated nutrients is a necessity (Wu et al. 2014). However, when wastewater is used, maintaining a single species population may be difficult. Christenson and Sims (2011) stated that monocultures are not found in wastewater systems, and Chen et al. (2015) concluded that the cultivation of mixed native species in wastewater is recommended to increase the stability of the cultivation system.

Microalgae can grow effectively in different types of industrial and municipal wastewater (Park et al. 2013, Quiroz Arita et al. 2015, Wu et al. 2014, Hidaka et al. 2014) and AD effluents (Hidaka et al. 2014, Morales-Amaral et al. 2015). In these varieties of wastewater, algae can reach 80–90% nitrogen and phosphorus removal (Christenson and Sims 2011, Chen et al. 2015). Wastewater can differ in composition between sources and also fluctuate across time periods (e.g., due to precipitation in the case of municipal wastewater or changing processes in the case of industrial wastewater). In addition, a wastewater-based medium can originate from very different steps in the treatment process; raw wastewater, water after aerobic treatment, and centrate from sludge dewatering have all been studied (Quiroz Arita et al. 2015). Digestate centrates from AD have much higher nutrient concentrations compared with wastewater, and the ammonium concentration often exceeds inhibitive levels for algae growth and needs to be diluted (Wu et al. 2014, Morales-Amaral et al. 2015).

When wastewater or digestate centrate are used to grow algae, a diversity of microorganisms coexist with the algae. The algae and bacteria demonstrate three kinds of interactions: in mutualism both partners benefit, in commensalism only one partner benefits, and in parasitism one partner benefits while the other is negatively affected. It has been discovered that algae and bacteria have a similar relationship to that of higher plants, with bacteria providing algae with inorganic carbon, nutrients, and vitamins and algae in return supplying organic carbon and oxygen to the bacteria (Figure 3). Indeed, bacteria may promote algae growth by 10–70% (Ramanan et al. 2016). On the other hand, it is also well-known that many bacteria can have a negative effect on algae, even to the point of causing cell lysis (Ramanan et al. 2016).
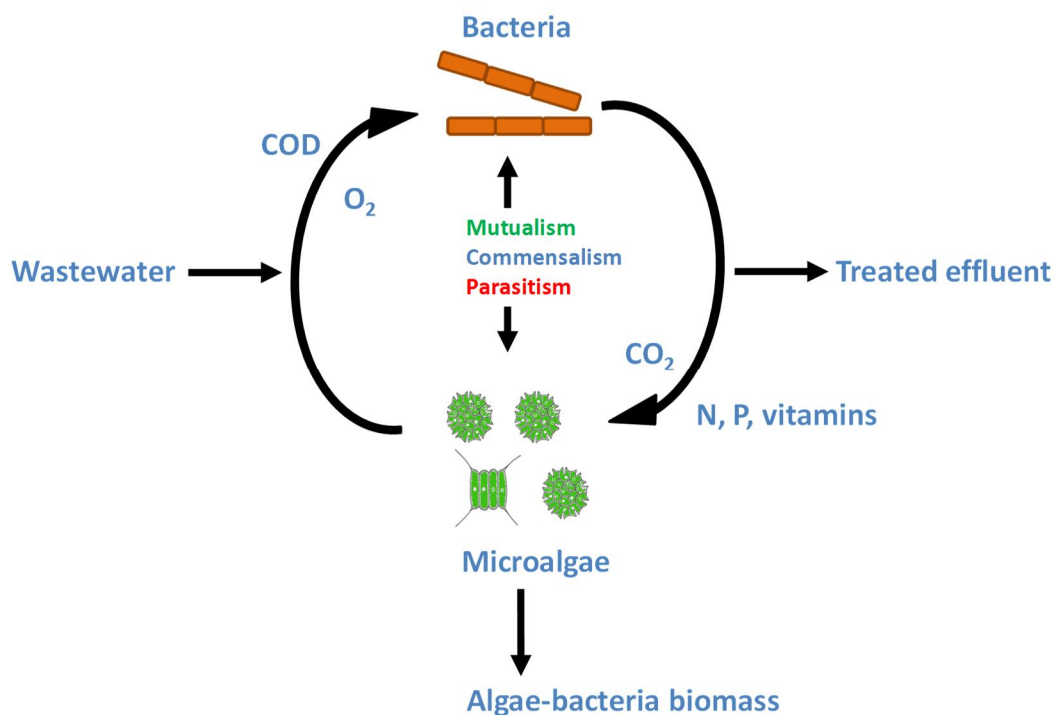

Figure 3. An illustration of algal-bacterial interactions in wastewater algae cultivation; redrawn after Passos and Ferrer (2014) and Ramanan et al. (2016)

The combination of low biomass concentration (~1 g L^{-1}) of microalgal culture and algae densities near water makes the harvesting of biomass challenging (Barros et al. 2015). Several methods have been developed to harvest microalgae, including gravity sedimentation, sedimentation following chemical flocculation/coagulation, auto- and bioflocculation, flotation, and electrical processes. Microalgae can also be harvested and/or further dewatered with centrifugation and filtration techniques (Mata et al. 2010, Milledge and Heaven 2013, Barros et al. 2015). Table 2 compares the solid concentrations reached with some of the most often used harvesting/dewatering methods. However, none of these technologies have been proven universally applicable, making harvesting and concentrating algal biomass one of the major energy consumers in algal biofuel process chain; this accounts for 20–30% of the total cost (Barros et al. 2015). Although the harvesting method for each case is dependent on the algae species and target products, simple sedimentation of algal biomass is considered to be among the most energy-efficient collection processes (Barros et al. 2015). Settling can be enhanced by recirculating a well-settling fraction of algae back to cultivation (Park et al. 2013) However, the TS concentration of algal biomass collected by settling remains below 3% (Table 2).

Table 2. Different harvesting/dewatering methods of microalgae and achieved solid concentrations (adapted from Christensson and Sims (2011), Milledge and Heaven (2013), and Barros et al. (2015))

Technology	Limitations	Biomass TS concentration (%)
Centrifugation	High energy consumption (electricity)	10–22
Filtration	High energy consumption (electricity)	2–27
Chemical precipitation/flocculation	Chemicals may inhibit anaerobic digestion	3–8
Flotation	Usually requires chemicals	3–6
Gravitational sedimentation	Slow, low biomass concentration	0.5–3

2.2 Microalgae as a feedstock for diesel fuels

The current interest in microalgae arises mainly from the ability of algae to accumulate intracellular lipids, which is a suitable raw material for biodiesel or renewable diesel production. Both of these diesel fuels are refined from lipid materials of biological origin, such as vegetable oils and animal fats. However, biodiesel and renewable diesel are entirely different products; biodiesel is made through a transesterification process with alcohol, while renewable diesel is produced using a hydrogenation process with hydrogen. Renewable diesel composition and properties are equal to those of fossil diesel fuels, but biodiesel quality depends more on the parent oil (lipid) composition, and its properties may not be at the level of fossil diesel (Knothe 2010).

Whether biodiesel or renewable diesel production is targeted, the successful cultivation of high-yield and lipid-rich algae strains is necessary (Collet et al. 2014). *Nannochloropsis* sp. (Ma et al. 2014), *Botryococcus* sp. (Cabanelas et al. 2015), and *Chlorella* sp. (Marjakangas et al. 2015) are examples of algal species extensively studied for diesel production purposes. Lipid content depends on the species and the environmental conditions in which the algae are cultivated. Lipid contents of 20–30% dry weight are commonly achieved (Taher et al. 2014), but the

reported range is wide, and up to 75% lipid contents have been achieved (Mata et al. 2010). High lipid contents are usually a result of algae cultivation in stress conditions (e.g., deficiency to nutrients, especially nitrogen or iron, or stress caused by high salinity). However, stress conditions may simultaneously cause the cessation of cell division, resulting in lower total biomass and lipid productivity (Mata et al. 2010).

The methods to extract intracellular lipids from microalgae biomass can be divided into wet and dry processes. In dry processes, the complete drying of the biomass with spray-drying, drum-drying, freeze-drying, or sun-drying is needed (Mata et al. 2010). From the dry biomass, lipids are extracted with mechanical and/or chemical methods (Mubarak et al. 2015). Commonly used solvents in chemical lipid extraction are n hexane, ethanol, and chloroform/methanol (Mubarak et al. 2015). As the high energy consumption related to the drying process has been demonstrated to make dry extraction a nonviable option (Collet et al. 2014, Quinn et al. 2014), wet extraction methods have been under development. Wet extraction could be possible from algae biomass having as low a TS as 7% (Taher et al. 2014, Chaudry et al. 2015, Mubarak et al. 2015).

Several life cycle studies have shown that diesel fuel production from microalgae is hardly viable. Collet et al. (2014) reported a net energy ratio (NER) of 1.07, Quinn et al. (2014) a NER of 1.03, and for algal biodiesel production the NER >1, meaning that more energy is consumed than produced. In the latter study, NER was improved to 0.68 when AD of the residual biomass was applied. However, the NER was still far from the current NER of 0.2 for conventional diesel (Quinn et al. 2014). Neither of these studies used wastewater as a nutrient source, but the nutrient supply was highlighted as one of the highest energy inputs to the system.

3   Pulp and paper biosludge

Although paper consumption is still increasing at the global level, the consumption in western countries has declined during the last decade (CEPI 2015, STATISTA). In addition, production has moved to new geographical areas with a higher paper demand and lower production costs. This is one of the reasons that the pulp and paper industry is searching for ways to diversify its production portfolio. The focus of development is toward biorefineries and a circular economy wherein a more efficient utilization of side-streams and wastes to create new products and/or energy is desired. One thus far underutilized waste stream in conventional pulp and paper mills is the biosludge produced in the wastewater treatment process.

Anaerobic treatment of pulp and paper industry wastewater is increasing, but the aerobic–activated sludge process (Figure 4) is still widely used (Meyer and Edwards, 2014). The activated-sludge process produces large volumes of biosludge (also known as secondary sludge or waste-activated-sludge). In addition to biosludge, primary sludge is produced, and it is usually incinerated. However, for biosludge, incineration is not favorable in terms of energy due to the low solid content of the sludge (Stoica et al. 2009). Furthermore, nutrient recovery, an integral part of the circular economy, is limited by the incineration option, as nitrogen is lost and phosphorus recovery from the ashes is challenging due to impurities (Reijnders 2014). Stoica et al. (2009) reported a yearly biosludge production of about 2900–4000 t/mill as TS for three Swedish pulp and paper mills, and sludge management can make up 50–60% of the costs of the pulp and paper mill wastewater treatment process (Mahmood and Elliot 2006, Meyer and Edwards 2014).
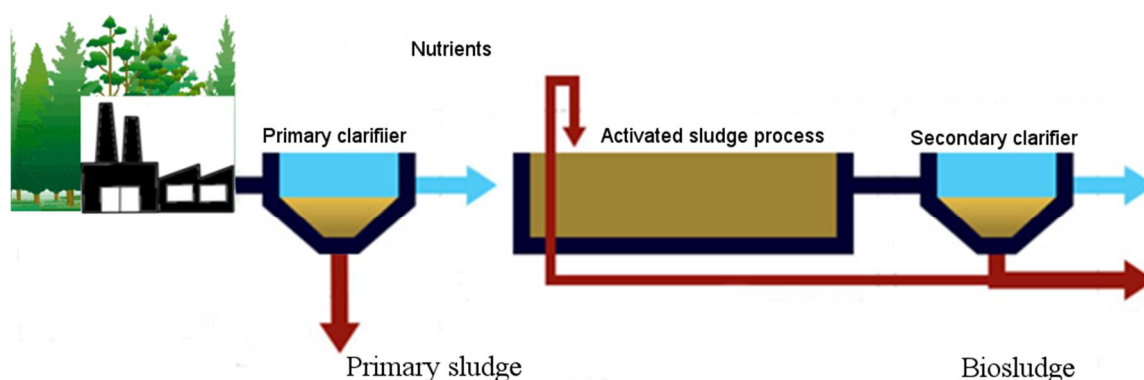

Figure 4. A simplified illustration of activated-sludge wastewater treatment in the pulp and paper industry.

Pulp and paper biosludge consists mainly of microbial cells from the activated-sludge process, associated extracellular polymeric substances, and the remaining lignocellulosic biomass from the pulp and paper process (Meyer and Edwards 2014). The characteristics of wastewater and biosludge may vary considerably between mills, pulping processes, raw materials used, and the wastewater treatment procedures used at the site (Bayr and Rintala 2012, Ekstrand et al. 2013). For this reason, it is impractical to describe pulp and paper biosludge in detail, and each process needs to be characterized separately. However, an important difference of biosludge compared to municipal wastewater treatment is that pulp and paper biosludge typically has a very high lignin and cellulose content. A lignin content of 36–50% and cellulose content of 19–27% TS have been reported, while these contents are usually <1% TS in municipal biosludge (Meyer and Edwards, 2014). Pulp and paper industry wastewater is often poor in nutrients (Bayr and Rintala 2012, Meyer and Edwards 2014), and nitrogen is added before the activated-sludge process to enable good biological performance of wastewater treatment. For this reason, biosludge may have a nitrogen content dozens of times higher compared to primary sludge (Meyer and Edwards 2014). The general characteristics of pulp and paper mill sludge are presented in Table 3.

Table 3. Characteristics of pulp and paper industry sludge compared to municipal biosludge. Adapted from Meyer and Edwards (2014).

Parameter	Municipal biosludge	Pulp and paper biosludge
Total dry solids (%TS)	0.8–1.2	1.0–2.0
Volatile solids (%TS)	59–68	65–97
Ash content (%TS)	19–59	12–41
N (%TS)	2.4–5.0	3.3–7.7
P (%TS)	0.5–0.7	0.5–2.8
pH	6.5–8.0	6.0–7.6
Heating value (MJ/kg — dry basis)	19–23	22–25
Carbohydrates (%VS)	17	0–23
Protein (%VS)	46–52	22–52
Lipids (%TS)	5–12	2–10
Cellulose (%TS)	~1	19–27
Lignin (%TS)	<0.1	36–50

4   Anaerobic digestion (AD)

AD is a biological process that produces methane and carbon dioxide (biogas) from organic matter. AD has been widely studied and is a proven technology for waste treatment and small-scale distributed energy production. For example, in Germany, over 10,000 biogas plants were in operation 2015 (IEA 2015). Common substrates for AD are sludge from wastewater treatment plants, manures, an organic fraction of municipal solid waste, crop residues, and energy crops. Different substrates can be digested either alone or together in co-digestion to improve the AD process or digestate quality (Mao et al. 2015).

Anaerobic degradation is often divided to four steps that all must work simultaneously: hydrolysis, acidogenesis (fermentation), acetogenesis, and methanogenesis (Mao et al. 2015, Figure 5). Each step requires its own specialized microorganisms, and each step can be rate-limiting for the whole AD process. For instance, volatile fatty acids (VFAs), which are intermediate products from acidogenesis, may inhibit methanogenic microorganisms in high concentrations. Hydrolysis or methanogenesis are usually the slowest phases of the degradation process, with the former affecting complex substrates and the latter influencing easily degradable substrates. Various pretreatment methods have been developed specifically to enhance the hydrolysis step of anaerobic degradation (Carrère et al. 2016).
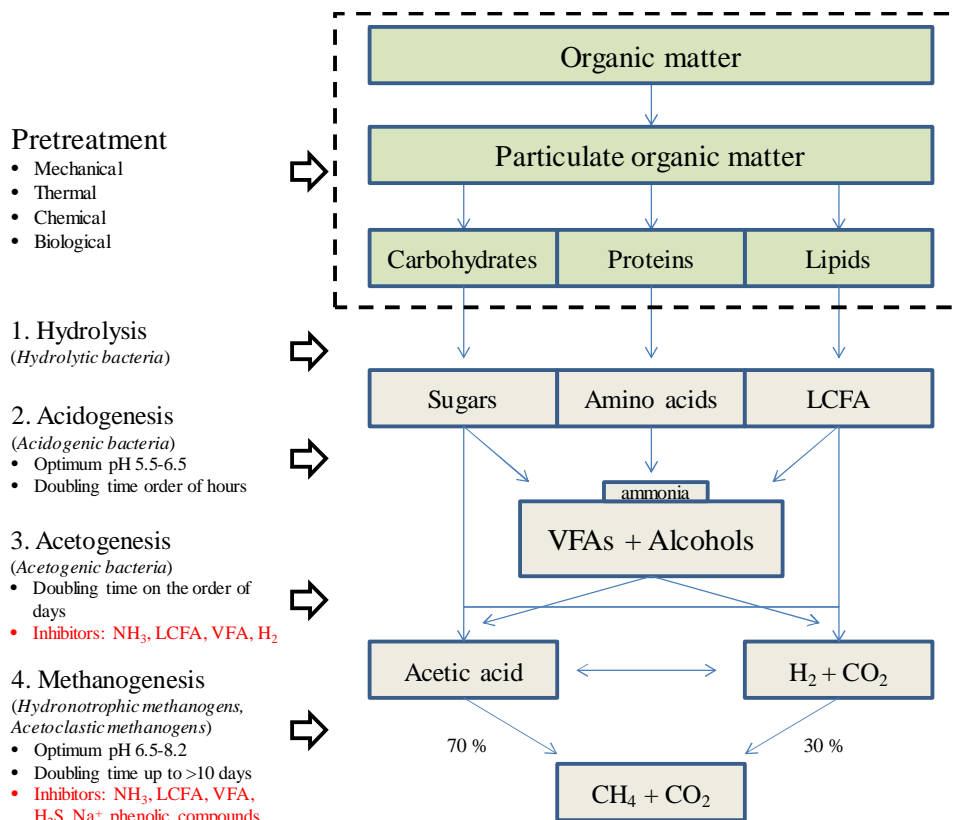

Figure 5. The microbiological steps of anaerobic degradation and the most common inhibitors related to the substrates (microalgae and biosludge from the pulp and paper industry) used in this study (Al Seadi et al. 2008, Chen et al. 2008, Mao et al. 2015).

AD is normally divided into three temperature ranges: psychrophilic (0–20ºC), mesophilic (20–40ºC), and thermophilic (45–70ºC) (Madigan et al. 2003). In practice, mesophilic and thermophilic processes dominate in full-scale applications, while there are small-scale digesters at the ambient temperature (e.g., in India). As methanogens with slow growth rates particularly benefit from temperature increases, thermophilic AD has the potential to offer a higher methane yield and a faster degradation rate compared with mesophilic and psychrophilic digestion (Mao et al. 2015). In addition, the requirements for hygienization are better accomplished in thermophilic than mesophilic AD. Despite the obvious benefits of the thermophilic process, it also has important drawbacks. Thermophilic AD is considered to be more sensitive to imbalanced operation and inhibitive substances (Angelidaki and Ahring 1993, Chen et al. 2008). Especially ammonia inhibition occurs more easily in thermophilic conditions, in part because a higher share of inhibitive unionized NH_3 forms at higher temperatures and pH (due to the lower solubility of CO_2) (Angelidaki and Ahring 1993, Chen et al. 2008).

Both mesophilic and thermophilic AD require considerable heating of the reactors in most climate zones, meaning a high energy input. With substrates that have low TS, such as microalgae and non-concentrated biosludge, what is actually being heated is mostly water. It is also possible that the surplus energy produced in thermophilic conditions is consumed by the higher energy demands of heating the process. AD in low temperatures (<20ºC) conducted by psychrophilic or acclimatized mesophilic microorganisms could be an interesting option, because no heating would be needed in temperate climate zones. In low temperatures, however, the metabolism of microorganisms is reduced; specifically, the hydrolysis step is considered rate-limiting (Halalsheh et al. 2011), meaning not only a lower methane yield but also a lower organic loading rate (OLR) and subsequently a longer hydraulic retention time (HRT) and a larger reactor size. Another drawback with low digestion temperature is the fact that methane is more soluble at a lower temperature, potentially causing losses in methane production and fugitive methane emissions if soluble methane is not recovered or used as a carbon source in downstream processes (Chen et al. 2015).

Biogas from AD is usually composed of about 60–70% methane and 30–40% carbon dioxide. In addition, biogas may contain nitrogen, hydrogen sulfide (H_2S) compounds such as siloxanes, and aromatic and halogenated hydrocompounds (Rasi 2009). After removing sulfur compounds, biogas can be utilized directly in heat and electricity production by a combined heat and power (CHP) unit. Optionally, biogas can be upgraded to biomethane by removing carbon dioxide, making it comparable to natural gas. Biomethane is a suitable fuel for gas-powered vehicles, or it may be injected into the natural gas grid when utilization options are similar to fossil natural gas. The methane production potential and the exact biogas composition vary according to the substrate used in AD, as the energy contents and (anaerobic) biodegradability of substrates are different. Fats have the highest methane production potential, followed by proteins and carbohydrates (Angelidaki and Sanders 2004).

The digestate from AD needs to be managed in an environmentally and economically sound way so that the whole process can be sustainable. In AD, nutrients such as nitrogen, phosphorus, and potassium present in the feedstock remain in the digestate. During the AD process, a significant proportion of the nitrogen turns into ammonium (NH_4^+), which is readily available for plants. Similarly, phosphorus is partly solubilized in anaerobic conditions to a liquid phosphate form (PO_4). The ability of the AD process to sustain nutrients and change their form

to one better suitable for plant uptake makes AD a prominent option for various nutrient recovery and recycling concepts in the circular economy. The simplest way to utilize digestate from AD is direct use in agriculture, but digestate can also be further refined to higher-quality liquid and solid nutrient products. Examples of digestate refining are nitrogen concentration via stripping (Huang et al. 2016) or reverse osmosis (Carter et al. 2015) and the chemical precipitation of phosphorus (Huang et al. 2015). The least valuable but still common use for digestate is directing the nutrient-rich liquid centrate to a wastewater treatment plant, where it causes a significant nitrogen load and an increased need for an external carbon source (Drosg et al. 2015).

OLR, HRT, and solid retention time (SRT) are the most important operation parameters of AD. OLR is the amount of organic matter introduced into digester volume per day. Too high loading may lead to overloading, as hydrolysis and acidogenesis produce more intermediate acids (VFAs) than slower processes such as acetogenesis and methanogenesis can consume. Eventual inhibition and acidifying of the process are irreversible (Mao et al. 2015). On the other hand, low OLR means low methane yield per reactor volume and a small amount of substrate being treated in given time (Al Seadi 2008, Mao et al. 2015). In AD, SRT is the average time that microorganisms (solids) stay in the digester, while HRT is defined as the time that substrate spends in the digester. SRT and HRT are the same in completely mixed reactors (such as a completely stirred tank reactor (CSTR)) without biomass circulation. In sludge retention reactors, typically used for wastewater and contact processes where solids are returned to the process, HRT and SRT are decoupled (Mao et al. 2015). OLR and HRT are usually connected so that increasing OLR decreases HRT and vice versa. A short HRT is desired as it allows for a smaller reactor size. However, too short of an HRT means improper degradation and even the wash-out of anaerobic microorganisms, leading to process failure (Figure 6). HRT cannot be shorter than the duplication time of the slowest reproducing anaerobic microorganism in the digester, which can be as slow as >10 d for methanogens. The suitable OLR, HRT, and reactor type depend on the substrate used. Microalgae and pulp and paper biosludge as substrates for AD are discussed in the next sections of this work.
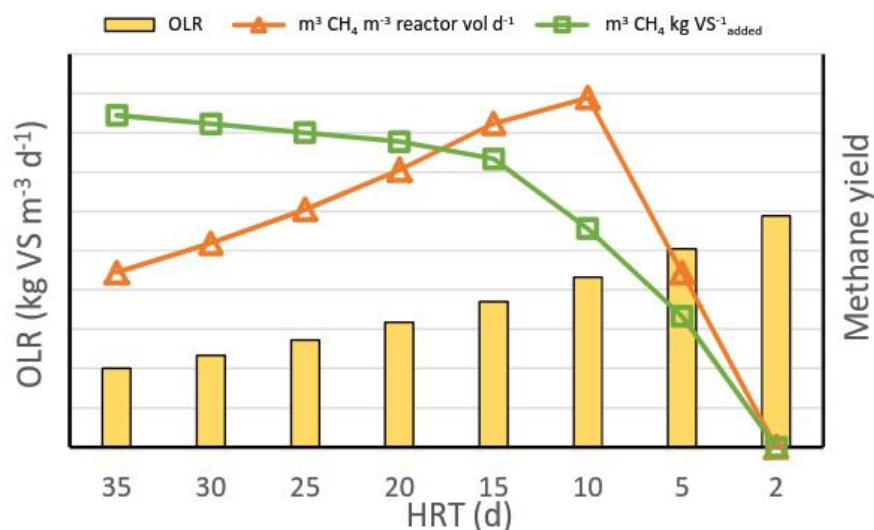
Figure 6. An illustration of the connections between HRT, OLR, and the volumetric and specific methane yields in a typical completely mixed anaerobic digester. Redrawn after Nges and Liu (2010) and Mao et al. (2015).

4.1 AD of microalgae and microalgae residues

Although AD is a mature technology, the detailed process is dependent on substrate characteristics; methane yield, degradability, and process stability may have considerable variation, and novel substrates should be studied before being applied at full scale. Microalgae have already been investigated for AD in the 1950s (Golueke et al. 1957), but it has not been until recently that more detailed AD studies with microalgae biomass have been conducted. There are several substrate-specific challenges for the AD of microalgae: a low solid content of the algal biomass (if no energy is consumed for dewatering); a difficult and slow degradability of many algae species, leading to low methane yields and requiring a long HRT (Ras et al. 2011, Alzate et al. 2012, Passos et al. 2014a); and a high nitrogen concentration of algae, potentially inhibiting the AD process (Sialve et al. 2009). In addition, if microalgae are cultivated in marine water, the salt may inhibit the AD process (Lakaniemi et al. 2011).

4.1.1 Degradability and Methane yield

Recent research has shown that the degradability and methane yield from microalgae are often low compared with theoretical values and other more conventional AD substrates (Passos et al. 2014a). The degradability and methane yield varies between algae species and is mainly affected by biomass composition (lipid, carbohydrates, and proteins) and the robustness of the algae cell wall. The biomass composition varies between species and growth conditions, but often microalgae biomass have high protein content, which may exceed 50% of the dry matter (Table 4).

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Table 4. Approximate composition end energy content of listed microalgae species on dry matter basis. Demirbas and Demirbas 2011 and Tibbets et al. 2015

Algae species	Protein (%)	Lipid (%)	Carbohydrate (%)	Ash (%)	Gross Energy (MJ kg^{-1})
Scenedesmus obliquus	50–56	12–14	10–17	n.a.	n.a.
Scenedesmus dimorphus	8–18	16–40	21–52	n.a.	n.a.
Acutodesmus dimorphus	28	19	39	15	21
Chlorella	53	16	25	6	24
Chlorella vulgaris	51–58	14–22	12–17	n.a.	n.a.
Chlorella pyrenoidosa	57	2	26	n.a.	n.a.
Phaeodactylum tricornutum	40	18	25	17	20
Nannochloropsis granulata	34	24	36	7	25
Nannochloropsis granulata	18	48	27	7	27
Botryococcus braunii	40	34	19	7	24
Botryococcus braunii	39	25	31	5	24
Neochloris oleoabundans	30	15	38	17	19
Porphyridium aerugineum	32	14	46	9	21
Tetraselmis chuii	47	12	25	16	20
Spirulina	56	14	22	8	23

Although the algae cell wall structure is not fully understood, it is known that some species completely lack a cell wall (e.g., *Dunaliella salina*) and some have a relatively easily degradable glycoprotein cell wall (*Clamydomonas* sp. *Tetraelmis* sp.). Most species, however, have developed a robust cell wall, with several layers of cellulose, hemicellulose, and recalcitrant compounds (*Chlorella* sp., *Nannochloropsis* sp., *Scenedesmus* sp.) (Gonzalez-Ferandez et al. 2011, Passos et al. 2014a). A tough cell wall efficiently hinders the degradability of the inner part of algae cells. Table 5 summarizes the biomethane potentials (BMPs) obtained from microalgae and microalgae residues in previous studies. Wastewater as a nutrient source will also add diverse bacterial fauna to the cultivation. Some studies suggest that methane production could be higher for algae biomass, including bacterial populations (Lü et al. 2013).

4.1.2 Ammonia inhibition

Ammonia inhibition is recognized as one of the most common reasons for inhibition in the AD process (Chen et al. 2008). Because microalgae are usually rich in protein, the biomass has a high nitrogen content. The optimal value for an AD substrate C/N ratio is suggested to be around 20–30 (Mao et al. 2015), with lower values exposing the process to ammonia inhibition. For algae, much lower C/N ratios of 5.3–10.2 (Elser et al. 2000, Yen and Brune 2007, Ehimen et al. 2009) and 4.4–5.6 (Ehimen et al. 2009, Park and Li 2012) have been reported for fresh biomass and residue cakes after lipid extraction, respectively. When lipids are extracted, a proportional part of the nitrogen in the residual biomass grows. Yen and Brune (2007) also found in practice that a low C/N ratio of microalgae sludge inhibited methane production. AD of algae in a thermophilic process has been reported to produce higher methane yields than in mesophilic digestion (Zamalloa et al. 2012), but thermophilic digestion possesses an increased risk of ammonia inhibition compared with mesophilic digestion.

Table 5. Biomethane potentials (BMPs) for microalgae and the impact of low-temperature pretreatments or lipid extraction on methane potential in recent studies.

Dominant species	Medium	BMP untreated/pre-treated (L CH$_4$ kgVS^{-1})	Pretreatment	Pretreatment efficiency (%)	Ref.
Chlorella sp.	Wastewater	78/126	2 h at 80°C	+61	Passos et al. 2016
Monoraphidium sp., *Scenedesmus* sp.	Wastewater	163	n.a.	n.a.	Gutiérrez et al. 2015
Stigeoclonium sp., *Monoraphidium* sp.	Wastewater	106/181	10 h at 95°C	+71	Passos et al. 2015
Ulothrix sp..	Wastewater	128–226	n.a.	n.a.	Van Den Hende et al. 2015
Ulothrix sp.	Wastewater	178/163	Freeze-thaw	–8	Van Den Hende et al. 2015
Chlamydomonas sp.	Wastewater	111/124	5 h at 55°C	+12	Passos et al. 2013
Chlamydomonas sp.	Wastewater	105/126	5 h at 55°C	+20	Passos et al. 2013
Scenedesmus sp.	Wastewater	410	n.a.	n.a.	Frigon et al. 2013
Scenedesmus sp.	Synthetic	306	n.a.	n.a.	Frigon et al. 2013
Chlorella sp.	Synthetic	318	n.a.	n.a.	Lü et al. 2013
Chlorella sp.	Synthetic, bacteria added	403	n.a.	n.a.	Lü et al. 2013
Scenedesmus sp.	Synthetic	n.a.	3 h at 70°C	+13	González-Fernández et al. 2012a
Scenedesmus sp.	Synthetic	n.a.	3 h at 90°C	+122	González-Fernández et al. 2012a
Scenedesmus sp.	Synthetic	n.a.	25 min at 70°C	+10	González-Fernández et al. 2012b
Scenedesmus sp.	Synthetic	n.a.	25 min at 80°C	+57	González-Fernández et al. 2012b
Scenedesmus sp.	Synthetic	180/240	Lipid extraction	+33	Keymer et al. 2013
Chlorella sp. (dried, frozen)	n.d.	443/283[a]	Lipid extraction	–36	Ehimen et al. 2009

[a] Estimated from figure, converted from given methane production per dry weight to methane production per VS using VS/TS share of 94.6%, n.d.= no data, n.a. = not applicable.

4.1.3 Salt inhibition

Avoiding fresh water consumption may be crucial for algal biofuel sustainability (Pate et al. 2011). When cultivated in marine water, algal biomass contains sea salt, which is known to have an inhibitive effect on AD (Chen et al. 2008). The salt concentration of marine-cultivated *Dunaliella tertiolecta* has been found to inhibit AD (Lakaniemi et al. 2011). On the other hand, successful AD in batch assays has been demonstrated with the marine alga *Dunaliella salina* up to 35 g L^{-1} of salinity, using adapted sediment inoculum collected from sea bed (Mottet et al. 2014). Also successful AD of *Tetraselmis* and saline wastewater have been reported (Asinari Di San Marzano et al. 1982, Lefebvre et al. 2007). In addition to affecting the AD process, salt likely limits the digestate's ability to be directly used as fertilizer due to the phytotoxic characteristics of sodium (McLachlan et al. 2004).

4.1.4 Anaerobic reactors for microalgae biomass

To date, all continuous AD studies with microalgae have been done in a laboratory or in a small pilot, as no full-scale algae biogas concepts exist. In most studies, CSTR-type reactors have been used. Methane production from algae in continuous or semi-continuous reactors has been studied using OLRs between 0.01 (De Schamphelaire and Verstraete, 2009) and 6 kg volatile solids (VS) m^{-3} d^{-1} (Yen and Brune, 2007, Park and Li, 2012). The OLRs at the high end of the range were reported to lead to overloading of the process (Yen and Brune, 2007, Ehimen et al. 2009, Park and Li 2012). For CSTR digesters, the low solid concentration of harvested algal biomass means short HRTs and/or low OLRs (Figure 7). Laboratory studies have typically had HRTs of 14–30 d (Ras et al. 2011, Passos et al. 2014a, Passos et al. 2014b). Ras et al. (2011) reported 63% higher methane yield from algal biomass with HRT of 28 d (240 L CH_4 kg^{-1} VS) compared to AD with HRT of 16 d.
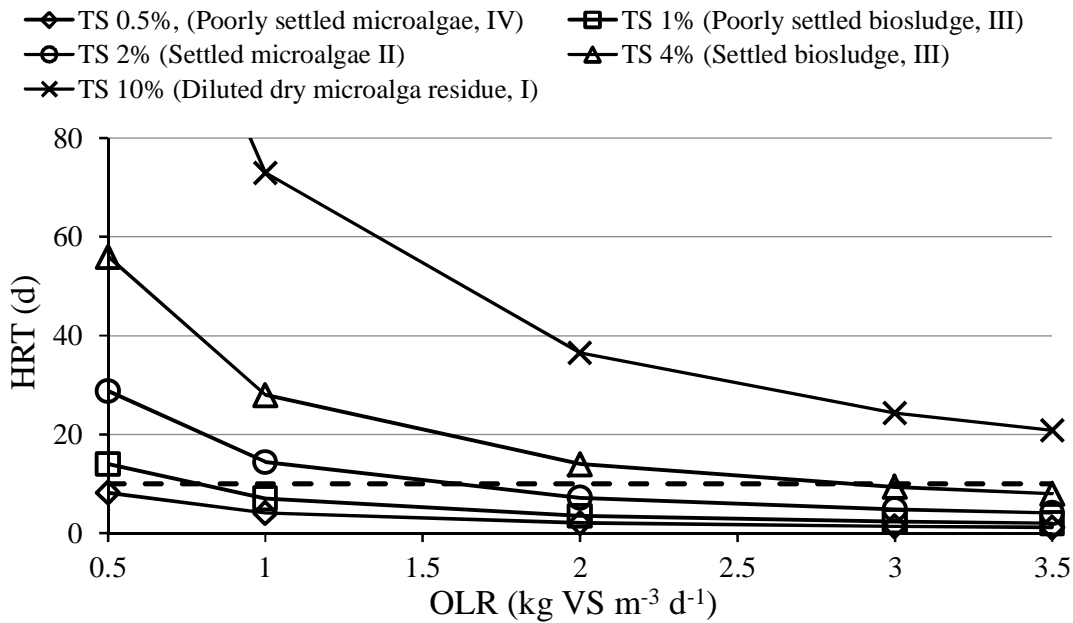
Figure 7. The relation between HRT and OLR in a completely mixed digester when using microalgal biomass or pulp and paper biosludge with typical TS concentrations. Roman numbers refer to the original papers in this thesis where the specific substrate was used.

4.2 AD of biosludge

AD of wastewater treatment plant sludge is a common practice, but it has rarely been applied to biosludge from the pulp and paper industry. Pulp and paper industry biosludge has very different characteristics compared to municipal biosludge, particularly its high content of lignocellulosic material that hinders anaerobic degradability.

In desired biorefinery concepts, nutrient recycling is as important a goal as methane recovery. However, pulp and paper biosludge often has a high concentration of cadmium, originating in the wood raw materials. If this sludge is used in AD, cadmium will also be present in the digestate and may exceed the limit concentrations set for fertilizer use. Hagelqvist (2013) reported a cadmium concentration of 2 mg kg^{-1} TS in pulp mill biosludge. In digestate, the concentration is likely to increase somewhat due to the degradation of solids. The legislative limit values for land-applied waste-originated products vary between 0.7 and 20 mg kg^{-1} TS in the European Union (Al Seadi and Lukehorst 2012), and are 0.8, 1.0, 1.5, and 2.0 mg kg^{-1} TS in Denmark, Sweden, Norway, and Finland, respectively. Other heavy metal concentrations in pulp and paper sludge are usually lower than the limit values (Hagelqvist 2013), but cadmium alone may make direct fertilizer use impossible. This means that the digestate needs to be further refined or alternative uses sought. Polishchuck et al. (2015) and Kouhia et al. (2015b) have suggested that pulp and paper biosludge digestate centrate be used for microalgae cultivation for biofuels and value-added products such as EPA in the biorefinery concept.

4.2.1 Methane yield, OLR, and HRT

Pulp and paper biosludge is characterized by a high lignin and cellulose content that originates from the lignocellulosic wood raw materials used in the pulping process. Lignocellulosic biomass is mainly composed of cellulose, hemicellulose, and lignin (Sawatdeenarunat et al. 2015, Carrère et al. 2016). The hydrolysis of these compounds decreases in the order of hemicellulose > cellulose > lignin (Carrère et al. 2016). Monlau et al. (2012) developed a model to predict the biomethane potential (BMP) of lignocellulosic biomass and found the lignin content to be the most important factor, with a strong negative impact on BMP. Methane yields from pulp and paper biosludge are usually very low because of its low degradability. Table 6 shows the BMPs reported for pulp and paper industry biosludge, and only a few BMPs exceeded 100 L CH_4 kg^{-1} VS. By comparison, the BMPs for municipal biosludge are often approximately twice as high, 200–250 L CH_4 kg^{-1} VS (Girault et al. 2012, Wang et al. 2014). As with microalgae biomass, pulp and paper biosludge also has a low TS content, meaning that in CSTRs it is not possible to increase OLR at the typical levels used in AD (2–3 kg VS m^{-3} d^{-1}) without HRT shortening too much for a complex substrate (Figure 7). Low OLR also means lower volumetric methane yield (Figure 6). Possibilities for enhancing the degradability of pulp and paper biosludge by pretreatments applied prior to the AD process are discussed in the next chapter.

Table 6. BMPs for pulp and paper biosludge and the impact of pretreatments on BMP

Pulp and paper biosludge	BMP temperature/duration	BMP (L CH$_4$ kg^{-1} VS) (untreated/pretreated)	Pretreatment	Pretreatment efficiency	Reference
Biosludge	55°C/22 d	67/72	Low-temperature 70°C	+7%	Bayr et al. (2013)
Biosludge	55°C/22 d	67/97	Thermal 150°C	+45%	Bayr et al. (2013)
Biosludge	55°C/22 d	67/68	Ultrasound	+1%	Bayr et al. (2013)
Biosludge	55°C/22 d	67/11	Alkali (NaOH)	-84%	Bayr et al. (2013)
Biosludge	55°C/22 d	67/0	Acid (HCl)	-100%	Bayr et al. (2013)
Biosludge	55°C/22 d	67/66	Enzyme	-1%	Bayr et al. (2013)
Biosludge (BCTMP/TMP)	36°C/28 d	88/96	Alkali (NaOH)+Ultrasound	+9%	Park et al. (2012)
Biosludge (mechanical)	35°C/20 d	118	n.a.	n.a.	Karlsson et al. (2011)
Biosludge (sulfite)	35°C/20 d	103	n.a.	n.a.	Karlsson et al. (2011)
Biosludge (kraft)	35°C/20 d	69–117	n.a.	n.a.	Karlsson et al. (2011)
Biosludge (CTMP/kraft)	35°C/20 d	43	n.a.	n.a.	Karlsson et al. (2011)
Biosludge (kraft/CTMP)	35°C/20 d	95/101	Ultrasound	+6%	Karlsson et al. (2011)
Biosludge	35°C/20 d	132/178	Enzyme	+35%	Karlsson et al. (2011)
Biosludge	35°C/20 d	132/196	Enzyme+ultrasound	+48%	Karlsson et al. (2011)

n.a. = not applicable

4.3 Pretreatments prior to AD

Microalgal biomass and pulp and paper biosludge share characteristics that make them difficult to degrade, the former because of a resilient cell wall and the latter because of a high lignin and cellulose content. Therefore, hydrolysis is a limiting factor in the AD process for both substrates. Hydrolysis can be enhanced by using suitable pretreatment methods. Pretreatment may increase the ultimate methane yield and/or hasten the rate of degradation (Carrère et al. 2016). Even if it has no impact on methane yield, increased degradation can allow for a shorter HRT and a smaller digester size. Other positive impacts, such as hygienization, better dewaterability, decreased viscosity, and more soluble nutrients in digestate may also follow pretreatment (Carrère et al. 2016). Pretreatments are often classified as mechanical, chemical, thermal, and biological methods (Carrère et al. 2016). Mechanical methods include size reduction by grinding or ultrasound pretreatment to increase the reactive surface area for microbes to attach. Typical chemical methods are an acid or base treatment prior to digestion in order to solubilize the substrate. Thermal treatments also aim to increase solubilization by particulate matter disintegration (Passos et al. 2014a). The terminology is not fully established, but thermal pretreatments are often classified as biological (55–70°C), low-temperature (80–100°C), hydrothermal (>100°C), and steam explosion (where temperature and pressure [~160°C, >6 bars] is applied and the pressure is quickly released, leading to cell rupture) (Passos et al. 2014a, Carrère et al. 2016).

The effect of pretreatments is very substrate-specific, as some methods are efficient for one substrate but not necessarily for another. The energy balance of pretreatment is the essential point when considering the viability of the various pretreatments. The energy gained after pretreatment should exceed the energy required (Carrère et al. 2016). However, the energy balance should be calculated from a life cycle perspective, because, as mentioned earlier, pretreatment may also provide other indirect energy benefits beyond methane yield. For example, a reduced transportation need due to increased solid reduction, higher solubilization of nutrients or better dewaterability of digestate can offer energy savings in downstream processes. In this thesis, low-temperature (<100°C) and freeze-thaw pretreatments for microalgae and hydrothermal pretreatment for pulp and paper biosludge were applied, and these are presented in the next sections.

4.3.1 Biological and low-temperature pretreatment

Low-temperature pretreatment is an attractive option because of the low energy demand compared to hydrothermal treatments and the possibility of taking advantage of excess heat (50–70°C) (e.g., from CHP units and industrial processes). Pretreatment at 55–70°C can be classified as a biological method because biological mechanisms are likely involved, but these are not yet well recognized (Carrère et al. 2016). Some experimental results suggest that at a temperature <70°C, solubilization is increased due to the hydrolyzation of EPS, while a temperature >80°C induces cell disruption and the release of intracellular matter (Passos et al. 2014a). Both the temperature and duration of pretreatment have an impact on its efficiency.

Exposure times from less than hour to several days have been tested (Passos et al. 2014a), and treatments as short as 3–4 h have been found to achieve >80% VS and chemical oxygen demand (COD) solubilization (González-Fernández et al. 2012a, Passos et al. 2013). Solubilization is an important factor for methane production, since hydrolysis is often the limiting step in the AD process.

Passos and Ferrer (2014) found that low thermal pretreatments of microalgae at 75ºC and 95ºC prior to AD obtained the best energy balance when compared with hydrothermal and microwave pretreatments. Low-temperature pretreatment of microalgae biomass (VS 5%) had an NER (calculated only for pretreatments) of 0.18, whereas it was 0.51 for hydrothermal treatment and >1 for microwave treatment, meaning a negative energy balance for the microwave treatment. However, the results of low-temperature pretreatment of microalgae have been contradictory, and they can be summarized as suggesting that the effectiveness is strongly related to the species of algae and especially to the cell wall structure. Another reason for contradictory results may be release of inhibitory substances, such as NH_3 during the pretreatment. Passos and Ferrer (2014) found that the diatom *Nitzschia* sp., with a strong cell wall, was not degraded even after low thermal pretreatment. Alzate et al. (2012) reported no effect or even decreased methane production after >12 h pretreatment of *Nannochloropsis*, *Scenedesmus*, and *Clamydomonas* at 55–60ºC. However, researchers such as Passos et al. (2013) showed that a 10 h low-temperature pretreatment increased BMP from *Clamydomonas* up to 62%.

Information about the role of bacteria in microalgae biomass on pretreatment efficiency is limited. However, it is known that parasitic/pathogenic bacteria excrete enzymes that degrade the algae cell wall, enhancing cell lysis (Ramanan et al. 2016). Indeed, bacteria-induced algae cell lysis has been reported to aid in the lipid extraction process (Lenneman et al. 2014).

4.3.2 Hydrothermal pretreatment

Various pretreatment methods have been screened to enhance the methane production of lignocellulosic biosludge, with hydrothermal pretreatments being among the most promising technologies (Wood et al. 2009, Saha et al. 2011, Bayr et al. 2013). Hydrothermal pretreatments for pulp and paper industry biosludge have been suggested to require temperatures above 150°C, which improves the hydrolysis of hemicellulose in particular (Hendriks and Zeeman 2009, Fernández-Cegri et al. 2012). Lignin solubilization begins at 160ºC, but inhibitive phenolic compounds and furans are also formed (Hendriks and Zeeman, 2009, Monlau et al. 2014, Carrère et al. 2016). Enhanced methane production from pulp and paper industry biosludge has been demonstrated at 150ºC (Bayr et al. 2013) and with microwave pretreatment at 75–175ºC (Saha et al. 2011). Several studies with municipal biosludge show that thermal pretreatment at temperatures <150ºC (e.g., at 121ºC) can also improve methane production (Bougrier et al. 2008, Carrère et al. 2010), but lower pretreatment temperatures are rarely studied for pulp and paper industry biosludge. A lower pretreatment temperature would reduce the input energy and prevent the formation of phenolic compounds from lignin.

4.3.3 Freeze-thaw pretreatment

The effect of freezing and subsequent thawing (hereafter referred as freeze-thaw) of substrate has been studied to condition digested wastewater sludge and found to improve sludge dewaterability and settleability (Örmeci and Veslind 2001, Hu et al. 2011, Gao 2011). An increased COD solubilization (Gao 2011) and methane yield (Montusiewicz et al. 2010) have also been reported. However, the published data about the impact of freeze-thaw on AD is not promising. Gao (2011) found that freezing also affected nutrient solubilization, with a 2.5-times increase of PO_4 concentration and 2–8 times increase of NH_3 concentration occurring after waste-activated sludge was treated for 24 h at -18ºC. Örmeci and Vesilind (2001) concluded that pretreatment leads to cell disruption and releases intracellular material, as indicated by elevated DNA, protein, and carbohydrate concentrations after the freeze-thaw treatment of activated sludge. As wastewater sludge and microalgal-bacterial biomass share the same unicellular physical properties, freeze-thaw could also be considered as a pretreatment method for algae, but no previous published literature exists. Freezing high volumes of a low solid substrate is energy-consuming and not likely to be a realistic pretreatment option, unless a method is discovered to take advantage of the winter season in cold-climate zones.

5   OBJECTIVES

The objective of the present thesis was to study AD of algal residues (after lipids have been extracted for diesel production), wastewater- or digestate-grown microalgae biomass, and pulp and paper industry biosludge, aiming to improve methane yield with pretreatments and reduce energy input with a low-cost anaerobic digester design. This main objective was divided into the following sub-objectives:

· To assess the feasibility of mesophilic and thermophilic AD processes to recover energy in the form of methane from a marine green microalga (*Nannochloropsis* sp.) residue cake after the extraction of lipids for renewable diesel production.

· To assess the feasibility of a low-cost AD process for wastewater-grown microalgae in different temperatures (15–35ºC).

· To assess the long-term performance of mesophilic AD of pulp and paper biosludge, aiming to produce methane and nutrient media for algal cultivation.

· To assess pretreatments (low-temperature, hydrothermal, and freeze-thaw) to improve algae and biosludge digestion.

· To assess the effect of low-temperature pretreatment on solubilization and methane production of native microalgae, comparing algae cultivated in synthetic medium, sterilized and non-sterilized wastewater and digestate from AD of pulp and paper biosludge.

6 MATERIALS AND METHODS

6.1 Experiments

An overview of the research conducted in this thesis is presented in Figure 8 and Table 7. AD was studied for microalgae residues after lipid extraction for renewable diesel production (I, number referring to original research paper), for microalgae grown in municipal wastewater (pilot-scale (II) and laboratory-scale cultivation (IV)), and in pulp and paper biosludge digestate (IV). Long-term AD of pulp and paper biosludge was studied for methane production and to produce digestate for an algae nutrient medium (III). Batch assays (I–IV) and reactor trials with CSTRs (I–III) and AVRs (II) were used. To enhance methane production from microalgae and biosludge, thermal pretreatments (II–IV) were studied.

Table 7. Objectives, substrates, pretreatments, and anaerobic digestion (AD) experiments.

Objective	Substrate	Pretreatment	AD Experiments	Temperature	Paper
Assess the feasibility of mesophilic and thermophilic AD for *Nannochloropsis* sp. residue after the extraction of lipids	Microalgae residues	Wet/dry lipid extraction	Batch, CSTR	35, 55°C	I
Assess the feasibility of pretreatments and AD in AVRs operated at low temperatures (15–35ºC) for wastewater-grown microalgae	Wastewater microalgae	Low-temperature 60°C, freeze-thaw	AVR, CSTR	8–20, 20, 35°C	II
Assess the feasibility of mesophilic AD of pulp and paper biosludge, aiming to produce methane and nutrient media for algal cultivation	Pulp and paper biosludge	Thermal 80–134°C	Batch, CSTR	35°C	III
Assess pretreatments (biological, low-temperature) to improve wastewater- and digestate-grown algae digestion	Wastewater & digestate microalgae	Biological & low-temperature 60–80°C	Batch	35°C	IV
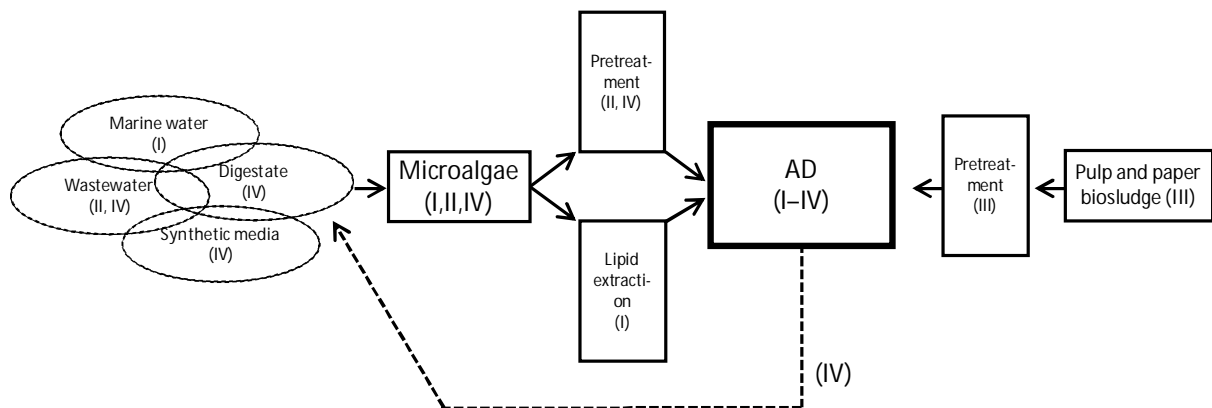
Figure 8. An overview of the experiments. Number indicates the original research paper.

6.2 Substrates and inocula

6.2.1 Microalgae (I, II, IV)

Marine microalga *Nannochloropsis* sp. residue cake was used to study methane recovery from microalgae residue after the extraction of lipids for renewable diesel production (I). *Nannochloropsis* biomass was cultivated in open marine water ponds in Israel.

Wastewater-grown microalgae biomass was used to study the effect of low-temperature thermal pretreatments and the performance of AVRs (II). The algal biomass was grown in an HRAP fed with primary settled sewage (Hamilton, New Zealand). The dominant species during the nine-month period was found to vary over time, consisting of *Pediastrum* sp., *Micractinium* sp., and *Scenedesmus* sp. For comparative experiments, the same algal biomass was used each time.

To study the effect of culture media on pretreatment efficiency (IV), the microalgal biomass was laboratory grown in 1 L glass bottles, using Jaworski's medium, municipal wastewater, and digestate (25% dilution with water) (centrifuged and filtered through GF/A) from AD of pulp and paper industry biosludge (III) as a growth medium. Microalgal biomass was a mixed population collected from Lake Pyhäjärvi (Tampere, Finland).

6.2.2 Pulp and paper industry biosludge (III)

This biosludge originated from a plant that treats pulp and paper industry wastewater. Incoming wastewater at this treatment plant included a minor fraction (<10% of volume) of municipal wastewater. During the 400-day study period, a new biosludge batch (~70 L in a 100 L container) was obtained every second month, for a total number of nine batches; all were stored at 7ºC before use.

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6.2.3 Inocula (I–IV)

Mesophilic inocula used in BMP assays and AD reactor trials were digestate from a mesophilic sewage sludge digester at the municipal wastewater treatment plant in Jyväskylä, Finland (I); effluent from an unmixed 5 m³ AVR that digested microalgae at ambient temperature (17–20ºC at the time of collection) in Hamilton, New Zealand (II); and municipal sewage sludge from the Viinikanlahti wastewater treatment plant (Tampere, Finland) (III, IV). Thermophilic inoculum (I) originated from a thermophilic digester handling sewage sludge and biowaste in Mustasaari, Finland.

6.3 Pretreatments

Lipid extraction from *Nannochloropsis* biomass (I) was performed either from air-spray dried algal biomass via methanol and hexane extraction or from wet algal biomass via ethanol and hexane extraction. To reduce the salt content of marine-originated algae, a part of the algal biomass was rinsed twice by mixing it with 10 times its volume of distilled water and subsequently separating fractions with a centrifuge (5 min at 3000 rpm).

For the low-temperature pretreatments, the algal biomass was incubated in 1 L bottles (II, III) or in 50 mL tubes (IV) in an incubator at a pretreatment temperature (60–80ºC) that was set for each experiment. For the freeze-thaw pretreatment, the algal biomass was placed in a 1 L (II) plastic bottle and kept at -20 ± 2ºC for 24 hours (h) and then melted at room temperature (20 ± 2ºC).

Thermal (105°C, 121°C, and 134°C) pretreatments were screened to improve the degradability of the biosludge. The biosludge was autoclaved (KSG Sterilisatoren GmbH) in loosely closed 1 L glass bottles with 500 mL of biosludge. The temperature reached 105°C, 121°C, and 134°C after 36, 45, and 50 min, respectively, while the pressure increased to 2.2 bars (gauge pressure). The set temperature was kept for about 20 minutes and then slowly cooled.

6.4 BMP assays (I-IV)

The setup of the BMP assays in each experiment is presented in Table 8. BMP assays were conducted in 60 (IV), 120 mL (I, III) with working volumes of 30 mL and 60 mL, respectively. $VS_{substrate}$:$VS_{inoculum}$ ratios of 0.2–1.0 were used. Bottles were closed with gas-tight caps, flushed with nitrogen, and incubated at the specific temperature of each experiment. Methane production from the inocula only was similarly determined and was subtracted from the methane production of samples.

Post-digestion experiments for digestate from reactors fed with microalgae residues (I) were done as BMP assays in 120 mL bottle, but only digestate was added. Post-digestion was done

to investigate remaining methane potential of organic matter that did not degrade during reactor run.

Table 8. Summary of BMP assays.

Substrate	Pretreatment	Volume (mL)	$VS_{subst}:VS_{inoc}$	Paper
Microalgae residue	Dry oil extraction	120	1	I
Microalgae residue	Wet oil extraction	120	1	I
Pulp and paper biosludge	Thermal	120	0.5	III
Wastewater microalgae	Low-temperature	60	0.2	IV
Digestate microalgae	Low-temperature	60	0.2	IV
Jaworski microalgae	Low-temperature	60	0.2	IV

6.5 Reactor trials (I–III)

CSTR and AVRs were used in the reactor trials. The reactors are illustrated in Figure 9, and the operational parameters used are summarized in Table 9.
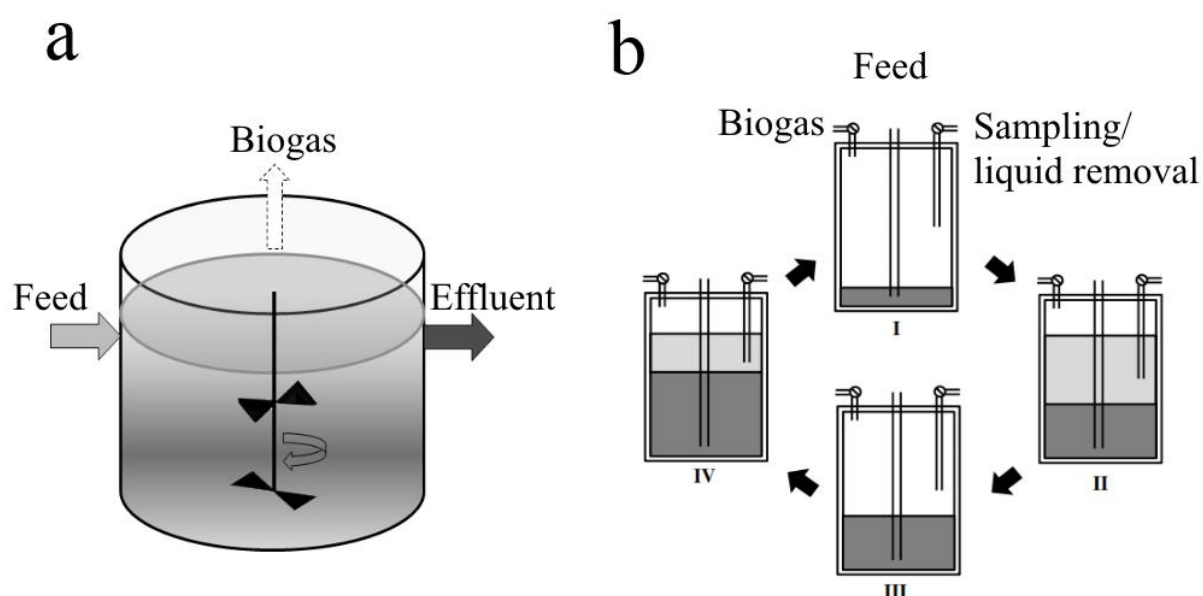

Figure 9. Design of **a**: completely stirred tank reactor (CSTR) and **b**: Accumulating-volume reactor (AVR).

Table 9. Substrates, pretreatments, reactors, and operational parameters (temperature, HRT, and OLR) used in reactor experiments.

Substrate	Pretreatment	Reactor type and volume	Temp. (°C)	HRT (d)	OLR (kg VS m^{-3} d^{-1})	Paper
Microalgae residue	None	CSTR, 5 L	35, 55	30–146	0.5–3.0	I
Wastewater microalgae	None, Freeze-thaw	CSTR, 2 L	37	14–16	1.0	II
Wastewater microalgae	None	AVR, 2 L	20, 37	128[a]	0.3–1.7	II
Wastewater microalgae	None, Freeze-thaw, Low-temperature	AVR, 20 L	(8-20)	91[a]	0.3–1.3	II
Pulp and paper biosludge	None, Thermal	CSTR, 5 L	35	10–20	0.5–2.2	III

[a] Solid retention time (SRT)

Semi-continuous CSTRs were used in the reactor trials. The reactor sizes were 2 L (II), 5 L (I), and 6 L (III), with working volumes of 1.5, 4, and 5 L, respectively. The CSTRs were fitted with outlets for biogas collection, feeding, and digestate withdrawal. Reactors were heated to 35°C (I, II, III) or 55°C (I) in an incubator (2 L and 5 L reactors) or using a heating mantle (6 L reactors) and mixed with mechanical stirrers. Biogas was collected into aluminum foil bags (5 or 10 L) Tesseraux (TECOBAG) via gas-tight tubes (Masterflex Tygon). Reactors were inoculated with inocula and fed every weekday (Monday to Friday). Prior to feeding, a volume of digestate approximately 10% less than the feeding volume was removed.

The AVRs were cylinder-shaped glass reactors with total volumes of 2 and 20 L. Each AVR was equipped with an outlet at the top for biogas collection; a tube was attached to the bottom of the reactor for feeding and solid sampling, and there was a vertically adjustable tube below the liquid level for liquid sampling and withdrawing liquids from the reactor. Reactors were unmixed. The ambient AVRs were placed outside in a water bath (~200 L) to moderate diurnal changes in temperature, which was monitored using a thermocouple connected to a data logger.

The AVRs were started by adding 200 mL (2 L reactors) or 2000 mL (20 L reactors) of inoculum. The AVRs were fed once a day on weekdays; subsequently, the liquid volume in the reactors increased gradually. The addition of algal biomass was continued until liquid volumes of ~1.8 (for the 2 L AVR) and ~10.5 L (for the 20 L AVR) were reached. The liquid fraction was then withdrawn, and feeding was resumed. Only one fill was monitored in the experiment with 20 L reactors.

6.6 Analyses and calculations

Analyses frequently used in this thesis are listed in Table 10. Specific analyses used in certain experiments only are described in detail in the original papers.

Table 10. List of analysis methods frequently used in experiments.

	Method	Paper
Methane content	Perkin Elmer Clarus 500 GC-FID gas chromatograph (Argon carrier; alumina column 30 m x 0.53 mm; oven, detector, and injector temperatures 100°C, 225°C, and 250°C, respectively)	(I)
	Portable gas analyzer (Geotechnical Instruments, GA2000)	(II)
	Perkin Elmer Clarus 500 GC-FID gas chromatograph (Helium carrier; Mol-Sieve 5A PLOT 30 m x 0.53 mm column; oven, detector, and injector temperatures 100°C, 250°C, and 230°C, respectively)	(III, IV)
	Shimadzu GC-2014 TCD gas chromatograph (helium carrier; ZB-WAX plus 30 m x 0.25 mm column; oven temperature 2 min 40°C, ramp 20°C/min to 160°C, ramp 40°C/min to 220°C, 2 min 220°C; detector and injector temperatures 250°C)	(III, IV)
Gas Volume	Water replacement	(I–III)
VFAs	Perkin-Elmer Autosystem XL gas chromatograph (helium carrier; PE FFAP column 30 m x 0.32 mm x 0.25 µm; oven 100–160°C (25°C/min); detector 225°C; injector 230°C)	(I)
	Ion chromatography (Hill Laboratories Ltd)	(II)
	Shimadzu GC-2010 FID gas chromatograph (helium carrier; ZB-WAX plus 30 m x 0.25 mm column; oven temperature 2 min 40°C, ramp 20°C/min to 160°C, ramp 40°C/min to 220°C, 2 min 220°C; detector and injector temperatures 250°C)	(III, IV)
TS and VS	APHA 2540	(I-IV)
COD	SFS 5504	(I)
	APHA 5220 D	(II–IV)
TKN	Tecator application note	(I)
	APHA 4000 N_{org} C	(II)
	EN 13654–1:2001	(III)
NH_4	Tecator application note	(I)
	APHA 4500-NH_3	(II–IV)
P_{tot}	APHA 4500-P E	(II)
	ICP-MS	(III)
Dissolved P	APHA 3125 B	(II)
PO_4	ISO 6878:2004(E)	(III)

The COD solubilization degree (S_D) of the biosludge after pretreatment was calculated using Eq. 1, as described in Donoso-Bravo et al. (2011).

$$S_D = \frac{sCOD - s_0COD}{tCOD - s_0COD} * \mathbf{100}$$
$$(1)$$

where sCOD is the soluble COD after pretreatment, s_0COD is the soluble COD in the untreated biosludge, and tCOD is the total COD of biosludge.

NH_3, the un-ionized fraction of NH_4^+ and the most inhibitive for the AD process, was calculated using Eq 2.

$$F_{NH_3} = \left(\mathbf{1} + \mathbf{10}^{(pk_w - pk_w - pH)}\right)^{-1} \qquad (2)$$

VS and TS removals in CSTR studies were calculated using Eq 3.

$$VS\ removal\ (\%) = \mathbf{100} * \frac{(VS_{in} - VS_{out})}{VS_{in}} \qquad (3)$$

VFA concentrations were converted to be equivalent with sCOD concentrations using the following values: acetic acid 1.066, propionic acid 1.512, iso-butyric and butyric acids 1.816, iso-valeric acid and valeric acids 2.036 (Ince, 1998).

BMPs are given as averages from triplicate assays unless otherwise mentioned. In reactor trials, methane yields from biosludge were calculated as weekly averages, using parallel reactors when applicable (II, III). All gas production results are given as normal temperature and pressure (NTP; 273 K, 1 bar), as the temperature and atmospheric pressure in the lab were monitored on a daily basis.

Statistical analysis of the results from BMP assays (IV) were done using IBM SPSS software (version 23). A one-way analysis of variance test followed by post hoc multiple comparison (Tukey HSD test) was conducted using 5% significance level after confirming normality (Kolmogorov-Smirnov test) and homogeneity of variances (Levene test).

The OLR and the HRT in reactor trials with CSTRs were calculated as weekly averages, either including the weekend (no feeding) (I) or excluding the weekend (II, III). When the weekend is excluded from the calculation, the OLR was actually higher and the HRT was shorter than the given weekly values.

The energy balance of AD and thermal pretreatments was estimated for microalgal biomass produced in an area of 1 ha and for pulp and paper biosludge produced in an average (3500 t TS a^{-1}) pulp and paper mill (2900–4000 t TS a^{-1}, Stoica et al. (2009)) according to Passos and Ferrer (2014), using Eq. 4–7. However, the following differences occurred in this study: a separate pretreatment reactor was included in the calculations, and the complete surface areas for reactor walls (pretreatment reactor and digester) were calculated. The parameters used to calculate energy balances are given in Table 11.

$$E_{i,heat} = \rho Q \gamma (\mathbf{T}_p - \mathbf{T}_a) + kA_p(\mathbf{T}_p - \mathbf{T}_a) - \rho Q \gamma (\mathbf{T}_p - \mathbf{T}_d)\phi + kA_d(\mathbf{T}_p - \mathbf{T}_a) \qquad (4)$$

where $E_{i,heat}$: input heat (kJ d^{-1}, results given in MWh a^{-1}); ρ: density (kg m^{-3}); Q: flow rate (m^3 d^{-1}); γ: specific heat (kJ kg^{-1}°C^{-1}); T_p: pretreatment temperature; T_a: ambient temperature (yearly average from Hamilton, New Zealand); T_d: anaerobic digestion temperature; k: heat transfer coefficient (Wm^{-2}°C^{-1}); A$_p$: surface area of the pretreatment reactor wall (m^2); A$_d$: surface area of the digester reactor wall (m^2); and ϕ: heat recovery efficiency.

$$E_{i,electricity} = Q\theta + \mathbf{V}\omega \qquad (5)$$

where $E_{i,electricity}$: input electricity (kJ d^{-1}); Q: flow rate (m^3 d^{-1}); θ: electricity consumption for pumping (kJ m^{-3}); V: useful volume (m^3); and ω: electricity consumption for mixing (kJ m^{-3}$_{reactor}$ d^{-1}).

The energy balance (ΔE) and energy ratio (E_o/E_i) were calculated using Eq. 6 and 7.

$$\Delta E = E_o - (E_{i,heat} + E_{i,electricity}) \qquad (6)$$

$$E_o/E_i = E_o/(E_{i,heat} + E_{i,electricity}) \qquad (7)$$

The reactor volume used was calculated using an HRT of 30 d for CSTRs and a 20% larger volume than the working volume (head space). AVRs were assumed to require three times the volume of the CSTRs.

Table 11. Parameters used to calculate the energy balances.

	Unit	Value	Reference
Parameter			
Density of water (ρ)	kg m^{-3}	1000	Passos and Ferrer (2014)
Specific heat of water (γ)	kJ kg^{-1}°C	4.18	Passos and Ferrer (2014)
Heat transfer coefficient (k)	W m^{-2}°C	1	Passos and Ferrer (2014)
Heat recovery by heat exchanger (ϕ)	%	85	Passos and Ferrer (2014)
Electricity consumption for pumping (θ)	kJ m^{-3}	1800	Passos and Ferrer (2014)
Electricity consumption rate for mixing (ω)	kJ m^{-3}·d	300	Passos and Ferrer (2014)
Lower heating value of methane	kWh m^{-3}	9.94	Passos and Ferrer (2014)
Ambient temperature (New Zealand) (T_a)	°C	13.8	www.niwa.co.nz
Anaerobic digestion temperature (T_d)	°C	16, 35	this study
Pretreatment temperature (T_p)	°C	60; 80	this study
Biomass production and concentration			
Algae production	g VSS m^{-2}	5.9; 10; 20	Mehrabadi et al. (2016)
Biosludge production	t TS mill^{-1} a	3500	Stoica et al. (2009)
Algae TS	%	2; 4	this study
Biosludge TS	%	1.3; 4	this study
Methane yields			
Algae in CSTR (35°C; HRT 30 d)	m^3 CH$_4$ t^{-1} VS	220	this study
Pretreated (4 h, 60°C) algae in CSTR (35°C; HRT 30 d)	m^3 CH$_4$ t^{-1} VS	250	this study
Pretreated (3 h, 80°C) algae in CSTR (35°C; HRT 30 d)	m^3 CH$_4$ t^{-1} VS	264	this study
Algae in AVR (16°C; SRT 90 d)	m^3 CH$_4$ t^{-1} VS	180	this study
Pretreated (4 h, 60°C) algae in AVR (16°C; SRT 90d)	m^3 CH$_4$ t^{-1} VS	225	this study
Biosludge (TS 1.3%) in CSTR (HRT 14 d)	m^3 CH$_4$ t^{-1} VS	70	this study
Biosludge (TS 4.0%) in CSTR (HRT 14 d)	m^3 CH$_4$ t^{-1} VS	77	this study
Pretreated biosludge in CSTR (HRT 10 & 14 d)	m^3 CH$_4$ t^{-1} VS	138	this study

7 RESULTS AND DISCUSSION

7.1 Substrate characteristics

The characteristics of the substrates (microalgae and pulp and paper biosludge) used in the AD experiments are summarized in Tables 12 and 13.

7.1.1 Microalgae

Microalgae biomass originated as a dry powder after lipid extraction (algae residue) (I), as liquid biomass from a pilot-scale HRAP (II), or as a laboratory-scale algae cultivation (IV). The TS content of the algae residue was high; 80–91%, as the biomass was dried after extraction. On the contrary, the TS content of microalgae from a pilot-scale wastewater HRAP (Hamilton, New Zealand) collected by gravity settling (II) was only 1.7–1.9%. The algae biomass used in experiments with different growth media (IV) had a relatively low TS content of 0.3–0.6%, because the collection was not the aim of the study. Algae biomass concentration is in line with the TS of 1.0–2.4% (II) that has been previously reported for wastewater-grown microalgae harvested by gravity settling (Alzate et al. 2012, Passos et al. 2013). The nitrogen (65–70 g kg TS^{-1}) and phosphorus (10–14 g kg^{-1} TS) content of microalgae grown in wastewater was higher than is typically present in biosludge from wastewater treatment (N: 24–50 g kg TS^{-1}, P 5–7g kg TS^{-1} (Meyer and Edwards 2014)). This demonstrates the potential to concentrate and recover wastewater nutrients using microalgae.

Table 13 presents the BMPs of studied microalgae. The BMPs for microalgae without lipid extraction were between 154 and 273 L CH_4 kg^{-1} VS. There is a notable variation in BMPs reported for microalgae biomass in the literature. For instance, rather low BMPs of 78–227 L CH_4 kg^{-1} VS for wastewater-grown, untreated microalgae have been recently reported (Gutiérrez et al. 2015, Passos et al., 2015a, Van den Hende et al., 2015, Passos et al. 2016). On the other hand, Frigon et al. (2013) reported a BMP as high as 410 L CH_4 kg^{-1} VS for *Scenedesmus*. In this study, clear difference in BMPs between algae grown in different media (synthetic, wastewater and digestate) was found that could explain the varying BMPs reported in literature. The precise reason for the high variations in algae BMPs remains to be determined, but it is likely that the growth media affects the algae cells' characteristics and cell wall structure.

For algae residues from lipid extraction, BMPs were 194 L CH_4 kg^{-1} VS for dry-extracted and 482 L CH_4 kg^{-1} VS for wet-extracted biomass (Table 13). Clearly, the higher (+148%) BMP for wet-extracted alga residue shows that the extraction method may have an important effect on algal residue methane production. However, the reason for the difference in BMP requires further study, and the role of the solvents used for extraction cannot be completely excluded.

7.1.2 Pulp and paper biosludge

Pulp and paper biosludge was collected from the secondary clarifier of a full-scale pulp and paper mill wastewater treatment plant, with nine collected samples covering a period of one year. The original TS content of the biosludge was 0.7–1.5%, but with a 24 h additional settling step, the TS content increased to 4.3%. The original TS content of biosludge (III) is in the range (1.0–2.0%) reported earlier for biosludge from pulp and paper wastewater treatment (Meyer and Edwards, 2014). Although the settling properties of biosludge varied, the higher TS content after an additional settling step indicates that solid separation in wastewater treatment process could be enhanced.

The nutrient composition of pulp and paper biosludge varied over the course of the year. The highest phosphorus content was seven times higher than the lowest (1.2–8.6 g kg^{-1} TS), and the highest nitrogen content was double that of the lowest (41–81 g kg^{-1} TS). This may be because of varying raw materials in pulp and paper production, but more likely it is a consequence of nutrient addition to the biological wastewater treatment process.

The BMPs of the biosludge samples were 85–102 L CH$_4$ kg^{-1} VS. Low BMPs in biosludge have already been widely reported (Meyer and Edwards, 2014); Bayr et al. (2013) found BMPs of 50–100 L CH$_4$ kg^{-1} VS, and Karlsson et al. (2011) reported slightly higher potentials (100–200 L CH$_4$ kg^{-1} VS) for six different varieties of pulp and paper industry biosludge, but their experiment used long incubation times (89–114 d). Pulp and paper industry biosludge BMPs remain low mostly due to the high lignin concentration of the substrate (about 44% of TS in the present study).

Table 12. Characteristics of substrates.

	TS	VS/TS	COD		N$_{tot}$	P$_{tot}$	
	(%)	(%)	(mg L^{-1})	pH	(g kg TS^{-1})	(g kg TS^{-1})	Paper
Microalgae residue, dry-extracted	91	73	n.a	6.6	70	n.d.	I
Microalgae residue, wet-extracted	80	83	n.a	6.5	n.d.	n.d.	I
Wastewater microalgae	1.8	72	20	6.5	65	10–14	II
Pulp and paper biosludge	1.1–4.3	65–78	12–35	7.2–7.4	44–81	1.2–8.6	III
Wastewater microalgae	0.57	87	6.7	6.8	n.d.	n.d.	IV
Digestate microalgae	0.34	77	3.4	6.3	n.d.	n.d.	IV
Jaworski microalgae	0.43	89	4.7	7.1	n.d.	n.d.	IV

n.d. not determined, n.a. = not applicable

Table 13. BMPs of the studied microalgae and pulp and paper biosludge and the effect of pretreatments on COD solubilization (S_D) and BMPs. Standard deviations are in parentheses when applicable.

Dominating Species	Cultivation media	BMP untreated/pre-treated (L CH$_4$ kgVS^{-1})	Pretreatment	COD solubility S_D (%)	BMP change after pretreatment (%)	Paper
Nannochlorosis residue, dry-extracted	Synthetic, marine	194 (8)	n.a.	n.a.	n.a.	I
Nannochlorosis residue, wet-extracted	Synthetic, marine	482 (34)	n.a.	n.a.	n.a.	I
Pediastrum sp., Microactinum sp.	Wastewater	273 (4)	n.a.	n.a.	n.a.	II
Pediastrum sp., Microactinum sp.	Wastewater	179/221[a]	3.8 h, 50–57°C	11	23	II
Pediastrum sp., Microactinum sp.	Wastewater	179/227[a]	Freeze-thaw	18	27	II
Scenedesmus sp., Coelastrum sp.	Synthetic (Jaworski)	252 (8)/280 (8)	3 h 80°C	13	11	IV
Scenedesmus sp.	Digestate	154 (2)/173 (2)	3 h 80°C	10	12	IV
Scenedesmus sp.	Sterile digestate	182 (10)/213 (6)	3 h 80°C	11	17	IV
Scenedesmus sp., Coelastrum sp.	Wastewater	222 (10)/259 (3)	3 h 80°C	12	17	IV
Scenedesmus sp., Coelastrum sp.	Sterile wastewater	236 (2)/292 (6)	3 h 80°C	11	24	IV
Pulp and paper biosludge	n.a.	85–102[b]	n.a.	n.a.	n.a.	III
Pulp and paper biosludge	n.a.	66 (1)/60 (7)	2 h 80°C	6	-9	III
Pulp and paper biosludge	n.a.	66 (1)/92 (4)	20 min 105°C	11	39	III
Pulp and paper biosludge	n.a.	66 (1)/107 (4)	20 min 121°C	14	62	III
Pulp and paper biosludge	n.a.	66 (1)/124 (4)	20 min 134°C	22	88	III

n.a. = not applicable, [a] at 20°C, [b] adapted inocula

7.1.3 The effect of pretreatments on biomass solubility and BMPs

For different microalgae biomasses, biological (50–60°C), low-temperature (80°C), and freeze-thaw pretreatments were applied to improve degradability and methane production. For biosludge samples, low-temperature (80°C) and thermal pretreatments were studied (105–134°C). The pretreatments used and their impact on the COD solubility and BMPs of algae and biosludge substrates are summarized in Table 13.

All pretreatments increased microalgae solubilization, with an S_D (COD-based) of 11% after biological pretreatment (3.7 h, 50–57°C) of *Pediastrum*-dominated wastewater-grown microalgae (II). Interestingly, the highest S_D (18%) for *Pediastrum* was achieved with freeze-thaw pretreatment. Low-temperature (3 h, 80°C) pretreatment of *Scenedesmus*-dominated microalgae biomass provided an S_D of 10–13% (IV). For pure culture of *Chlorella*, grown in synthetic Jaworski's medium, the S_D was clearly lower (5–7%) compared with *Chlorella* biomass grown in wastewater or digestate (S_D 9–12%). However, no difference between sterilized and non-sterilized wastewater was found. This indicate that the increased solubilization of *Chlorella* in low-thermal pretreatments could be related more to different cell composition due different growth media (e.g. Jaworski and wastewater) than to the bacteria in the growth medium. The S_D:s are comparable with other studies with similar kind of pretreatments applied for same algae species; Alzate et al. (2012) found an S_D of 9–11% after 12–24 h at 55°C for *Scenedesmus*-dominated biomass, and González-Fernández et al. (2012a, 2012b) found an S_D of 6–8% after 0.5–1 h pretreatment at 70–90°C, also for *Scenedesmus* biomass. Freeze-thaw pretreatment has been studied earlier for wastewater sludge. Hu et al. (2011) showed solubilization degrees of ~3–11% for wastewater sludge. Compared with their results, the S_D (18%) for algae in this study was relatively high.

The solubility is increased due to enhanced hydrolysis. The pretreatments seemed to improve protein degradation in particular, since the protein content of the pretreated and digested biomass was lower than that of the untreated biomass (II). This supports the findings by Passos et al. (2016), who also reported that thermal pretreatments can aid protein hydrolysis. Freezing most likely breaks algae cells, and intracellular liquids are then released. In addition to increased solubilization, freeze-thaw pretreatment clearly improved the settling properties of algae biomass (II). Hu et al. (2010) showed a similar improvement in the settling properties of wastewater sludge following freeze-thaw pretreatment.

The literature results are conflicting with regards to improved methane production following increased solubilization. For microalgae biomass, Passos et al. (2013 and 2015) showed that increased solubilization correlated well with enhanced methane production; on the other hand, Alzate et al. (2012) and Van den Hende et al. (2015) have reported increased solubility after pretreatment but no impact on methane production. In this study, however, pretreatments increased both solubility and BMPs. BMPs were enhanced by 11–27%, with freeze-thaw pretreatment having the highest S_D and also the highest increase in BMP. The impact of pretreatment on algae BMPs is in agreement with other studies, although once again the literature varies widely. González-Fernández et al. (2012a and 2012b) found a 3 h pretreatment at 70°C improved BMP by 13% and 0.5 h at 80°C by 57% for *Scenedesmus* biomass. Recently,

Passos et al. (2016) reported a 61% increase after 2 h pretreatment at 80°C for wastewater-grown algae. Alzate et al. (2012) observed moderate (4–5%) or even decreased (up to -13%) BMPs for algae after 12 or 24 h treatment at 55°C. The most efficient pretreatment in this study, freeze-thaw pretreatment, has been earlier reported to increase methane production from wastewater sludge (Montusiewicz et al. 2010). However, Van den Hende et al. (2015) found freeze-thaw pretreatment to have no impact on microalgae biomass. It is likely that freeze-thaw pretreatment cannot break the cell structure of all algae species, but worked for *Pediastrum* biomass used in this work (II), as this speceis have a relatively weak cell wall.

Pretreatment of biosludge was studied using treatment times and temperatures of 2 h at 80°C and 20 min at 105, 121, or 134°C (III). The S_D increased with all four tested treatment temperatures (13). The highest S_D of 22% was achieved after treatment at the highest tested temperature, 134°C, while the S_D was lowest (6%) after treatment at 80°C. The BMPs increased correspondingly with increased S_D; the BMP after pretreatment at 134°C was 88% higher (124 L CH_4 kg^{-1} VS) than that for sludge without pretreatment (66 L CH_4 kg^{-1} VS). The difference in methane production between untreated and pretreated biosludge was even more noticeable during the first 10 days of assay, when 140% (treated at 134°C) and 100% (treated at 121°C) more methane (108 and 90 L CH_4 kg^{-1} VS, respectively) was formed compared with untreated biosludge (45 L CH_4 kg^{-1} VS). The BMP of biosludge did not increase; in fact, it decreased 9% after pretreatment at 80°C.

The present study shows that enhancement in pulp and paper biosludge BMPs were achieved with pretreatments at 105–134°C. The increased solubilization and higher BMPs are in accordance with earlier findings, with a 55–280% (Wood et al. 2009) and 68% (Saha et al. 2011) increase in BMPs after thermal treatments at 170–175°C. However, the results in this study were achieved at much lower temperatures. The lignin and cellulose concentrations were not measured after pretreatments, so it is unclear whether the increased solubility was due to the breakdown of these compounds or the breakdown of a microbial biomass. As microbial biomass is a major component of biosludge, it is likely that increased solubility at relatively low-temperature pretreatments also originated from microbes. However, Saha et al. (2011) observed that soluble sugars from pulp and paper biosludge increased significantly when the pretreatment temperature exceeded 100°C, suggesting the solubilization of cellulose to some extent.

7.2 Reactor trials

Semi-continuous AD reactor trials were used to study the impact of loading and retention times on methane production and the stability of the AD process. CSTRs were used for all substrates and, in addition, wastewater algae were studied in AVRs. Mesophilic and thermophilic AD conditions were compared for algae residues, pulp and paper biosludge was studied in a mesophilic condition, and wastewater microalgae were studied in psychrophilic (ambient, 20°C) and mesophilic conditions. Table 14 summarizes the methane yields and operation parameters in reactor trials. Figures 10 shows methane yields in CSTR experiments, and Figure 11 shows the methane production in AVR experiments.

Table 14. Substrates, operating parameters, methane yields, and VS removals in reactor trials. Standard deviations are in parentheses when applicable.

Substrate (pretreatment)	Reactor	OLR (kg VS m^{-3} d^{-1})	HRT (d)	Methane yield (L CH_4 kg VS^{-1})	(L CH_4 kg FM^{-1})	VS removal (%)	Paper
Microalgae residue	CSTR 35°C	2	36	156 (11)	104 (8)	42 (3)	I
Microalgae residue (rinsed)	CSTR 35°C	3	30	128 (3)	85 (2)	33 (2)	I
Microalgae residue (rinsed)	CSTR 55°C	1.5	61	220 (22)	146 (15)	58 (2)	I
Wastewater microalgae	AVR, ambient (8–21°C)	1.7 à 0.3	128[a]	83 (11)	1.2 (0.2)	24 (2)	II
Wastewater microalgae	AVR 20°C	1.3 à 0.3	91[a]	100–106	1.4–1.5	34–37	II
Wastewater microalgae (3.8 h, 50–57°C)	AVR 20°C	1.3 à 0.3	91[a]	133–138	1.9–1.9	41–41	II
Wastewater microalgae (Freeze-thaw)	AVR 20°C	1.3 à 0.3	91[a]	155 (25)	2.2 (0.4)	42 (1)	II
Wastewater microalgae	CSTR 35°C	1	15	179 (17)	2.1 (0.3)	32 (1)	II
Wastewater microalgae (Freeze-thaw)	CSTR 35°C	1	15	205 (20)	2.5 (0.3)	39 (1)	II
Pulp and paper biosludge	CSTR 35°C	1.9	14	78 (3)	2.1 (0.2)	10	III
Pulp and paper biosludge (20 min, 121°C)	CSTR 35°C	2.2	10	134 (13)	2.5 (0.3)	19	III

[a] solid retention time (SRT)

40

7.2.1 Lipid-extracted algae residues

Algae residues were studied in 183 d reactor trials with four parallel CSTRs (I). The results are shown in Table 15. The thermophilic process provided the highest methane yield from algae residues (220 L CH_4 kg^{-1} VS), which was 48% more than in the comparative mesophilic reactor (149 L CH_4 kg^{-1} VS) with the same OLR (1.5 kg VS m^{-3} d^{-1}) and HRT (61 d). However, this was the highest-loading thermophilic process that demonstrated a stable reactor performance; once the OLR reached 2 kg VS m^{-3} d^{-1} (HRT 46 d), methane production and the methane concentration of the biogas decreased rapidly (Figure 10a). In addition, the reactor showed clear signs of unstable operation via increased propionate concentration. This suggests that, in all likelihood, the unstable operation of the thermophilic reactor was caused by ammonia inhibition originating from the high nitrogen content (70 g kg TS^{-1}) of the residue cake.

In contrast to thermophilic operation, in mesophilic conditions, it was possible to increase OLR to 3 kg VS m^{-3} d^{-1} (HRT 30 d) without disturbances in the methane yield or VFA profiles. However, methane yields decreased from 174 to 128 L CH_4 kg^{-1} VS when HRTs were shortened from 146 to 30 d. At the same time, methane production from post-digestion increased from 13 to 42 L CH_4 kg^{-1} VS (Table 15), showing that algae residues were slowly degradable. Finally, wet-extracted residue cake was also studied in the mesophilic reactors, with OLRs increased to 4 kg VS m^{-3} d^{-1} (HRTs 21–23 d). After the introduction of wet-extracted residue cake, methane yields increased to 153 L CH_4 kg^{-1} VS (Figure 10a), confirming the higher BMP of wet-extracted biomass, tested in batch assays. However, the HRT of 22 d was also too short for wet-extracted biomass, as the methane yield from post-digestion increased to 93 L CH_4 kg^{-1} VS. This emphasizes that it is important to manage residual methane production from digestate to avoid methane emissions with short HRTs.

Methane yields in this study were slightly higher than the 130 L CH_4 kg^{-1} VS that Park and Li (2012) reported with similar OLRs for wet-extracted *Nannochloropsis* residues in a mesophilic process (OLR 2 kg VS m^{-3} d^{-1}, HRT 40 d). These authors' methane yields decreased to almost to zero when the OLR was increased from 2 to 3 kg VS m^{-3} d^{-1}.

Parallel mesophilic reactors were operated with rinsed and non-rinsed residue cake. Rinsing was conducted to wash away the high salt content of marine microalgae biomass, which is known to potentially inhibit AD. However, the methane yields and operational parameters of the reactor fed with non-rinsed residue cake were comparable to the yields and operation from reactors fed with rinsed residue cake, although 5–16% lower methane yields were obtained after rinsing due to the loss of easily soluble organic matter. It can be concluded that inhibition caused by salt concentration (sodium concentration 2.9 g L^{-1}) in residue cake was insignificant or that the microbial population acclimated to increasing salt concentration during the reactor trial. The result is in agreement with findings by Mottet et al. (2014), who reported sodium concentration of 10.8 g L^{-1} not to affect digestion of marine algal biomass when adapted or acclimated inoculum was used, but to inhibit methane production with non-acclimated inoculum. On the other hand, Lakaniemi et al. (2011) found salt inhibition in AD of marine alga *Dunaliella tertiolecta* with a sodium concentration as low as 2.1 g L^{-1}. Even when salt concentration does not affect the AD of marine algal residue, salt may well limit the use of the digestate as a fertilizer.

Table 15. Methane yields and characteristics of digestates during reactor trials with algae residues.

	Reactor											
	Mesophilic				Mesophilic, rinsed		Mesophilic, rinsed				Thermophilic, rinsed	
OLR (kg VS m^{-3} d^{-1})[a]	0.5	1	2	4[b]	0.5	1	1.5	2	3	4[b]	1.5	2
HRT (d)[a]	146	82	36	21	183	91	61	46	30	23	61	46
Days	1–71	72–119	120–149	150–176	1–50	51–98	1–71	72–119	120–149	150–183	1–71	72–134
Methane yields												
(L CH_4 kg^{-1} VS_{added})	208 (15)	180 (6)	156 (11)	158 (35)	174 (6)	165 (10)	149 (10)	147 (13)	128 (3)	153 (38)	220 (22)	155 (26)
(m^3 CH_4 t^{-1} FM)	134 (16)	120 (4)	104 (8)	104 (23)	115 (4)	109 (7)	99 (7)	98 (9)	85 (2)	108 (27)	146 (15)	103 (17)
(m^3 CH_4 $m^{-3}_{liquid\ vol}$)	0.07 (0.01)	0.13 (0.01)	0.22 (0.02)	0.45 (0.10)	0.06 (0.01)	0.12 (0.01)	0.15 (0.02)	0.21 (0.02)	0.27 (0.01)	0.46 (0.12)	0.24 (0.03)	0.23 (0.03)
Methane concentration (%)	59 (2)	59 (2)	60 (4)	59 (4)	62 (6)	60 (2)	61 (2)	61 (2)	61 (1)	61 (5)	54 (3)	51 (3)
VS removal (%)	69 (2)	56 (3)	42 (3)	42 (1)	76 (3)	64 (3)	57 (3)	41 (2)	33 (2)	34 (1)	58 (2)	41 (1)
Post-methane potentials												
30 d (L CH_4 kg^{-1} VS_{feed})	8 (1)	5 (1)	29 (2)	45 (2)	8–8	n.d.	15–16	20–20	23 (1)	66 (2)	20–21	30 (1)
100 d (L CH_4 kg^{-1} VS_{feed})	15 (1)	26 (2)	51 (3)	100 (4)	13–13	n.d.	27–28	36–38	42 (2)	93 (5)	44–46	38 (1)
30 d (m^3 CH_4 t^{-1}digestate)	0.6 (0.1)	0.4 (0.1)	2.1 (0.1)	3.7 (0.2)	0.7–0.7	n.d.	1.3–1.4	1.8–1.8	2.2 (0.1)	5.9 (0.2)	1.8–1.9	2.7 (0.1)
100 d (m^3 CH_4 t^{-1}digestate)	1.2 (0.1)	1.9 (0.1)	3.7 (0.2)	8.3 (0.3)	1.3–1.3	n.d.	2.4–2.5	3.3–3.4	3.9 (0.2)	8.3 (0.4)	4.1–4.3	3.5 (0.1)
Total methane production												
(L CH_4 kg^{-1} VS_{added})	223 (16)	206 (8)	207 (14)	258 (39)	187 (6)	n.d.	177 (11)	183 (14)	170 (5)	246 (43)	265 (24)	193 (27)
Digestate characteristics												
TVFA (mg L^{-1})	90	50	830	1570	50	20	40	220	100	3780	420	3750
SCOD (g L^{-1})	0.9 (0.1)	1.6 (0.1)	2.5 (0.1)	4.4 (0.1)	1.1 (0.1)	1.5 (0.2)	2.2 (0.1)	4.6 (0.1)	5.4 (0.2)	7.0 (0.3)	7.1 (0.1)	15.8 (0.5)
NH_4^+ (g L^{-1})	1.0 (0.1)	1.0 (0.1)	1.1 (0.1)	1.9 (0.1)	0.8 (0.1)	1.0 (0.1)	0.9 (0.1)	1.2 (0.1)	1.3 (0.1)	2.1 (0.1)	1.7 (0.1)	2.9 (0.1)
TKN (g L^{-1})	3.3 (0.5)	3.2 (0.5)	6.2 (0.2)	7.7 (0.3)	2.9 (0.8)	3.2 (0.6)	4.6 (0.2)	5.4 (2.0)	6.2 (0.2)	7.5 (0.4)	5.5 (0.3)	8.6 (0.2)
NH_4^+/TKN (%)	30	31	18	25	28	31	20	22	21	28	31	33
pH	7.4 (0.2)	7.2 (0.1)	7.2 (0.1)	7.4 (0.1)	7.4 (0.1)	7.3 (0.1)	7.4 (0.1)	7.4 (0.1)	7.4 (0.1)	7.5 (0.1)	7.7 (0.2)	7.9 (0.1)

[a] Calculated by daily feed volume and VS in semi-continuous digestion, [b] Fed with N2, n.d. = not determined.

7.2.2 Wastewater microalgae

The AD of gravity-settled microalgae biomass grown in a pilot-scale HRAP treating municipal wastewater was studied in three parallel semi-continuous CSTRs for 130 d and three parallel AVRs for 125–170 d (II). The CSTRs were fed with untreated and freeze-thaw pretreated microalgae biomass, while the AVRs were operated in mesophilic and psychrophilic temperatures (20ºC, ambient (8–21°C)), and untreated algae, freeze-thaw and biological pretreatments (at ~60ºC) were investigated.

CSTRs were operated with a constant OLR of 1.0 g VS L^{-1} d^{-1} and an HRT of 14–16 d. The methane yield from untreated wastewater-grown microalgae biomass dominated by *Pediastrum* sp. and *Micractinium* sp. was 179 L CH_4 kg^{-1} VS. Freeze-thaw pretreatment increased the methane yield by 14% (205 L CH_4 kg^{-1} VS) (Figure 10b).

The TS of algae biomass harvested by gravity settling was only 1.7–1.8%, which is rather low for conventional AD in CSTR. Indeed, the OLR used of 1.0 g VS L^{-1} d^{-1} could not have been increased without decreasing the HRT to <14 d. For this reason, AVRs that decoupled HRT and SRT and allowed longer degradation time for solids were used. Microalgae were digested in the AVRs at 20°C, 37°C, and ambient temperature (8–21°C). The methane yields were 101 L CH_4 kg^{-1} VS and 225 L CH_4 kg^{-1} VS at 20°C and 37°C, respectively (Figure 11a). The ultimate methane yields after post-digestion (no feeding) were 180 L CH_4 kg^{-1} VS at 20°C and 273 L CH_4 kg^{-1} VS at 37°C. The ambient temperature AVRs had a comparable methane yield to the AVRs at 20°C as long as the temperature remained above 18ºC, but the methane yield started to decline when the temperature decreased to 16ºC and almost ceased below 15ºC (Figure 11c). The methane yield of the ambient temperature AVRs averaged 83 L CH_4 kg^{-1} VS (16–18ºC) during the fill, and the ultimate methane yield was 121 L CH_4 kg^{-1} VS post-digestion.

Both freeze-thaw and biological pretreatment of microalgae enhanced the methane yields at 20°C. Biological pretreatment (60°C) enhanced methane production by 32% (136 L CH_4 kg^{-1} VS) and freeze-thaw pretreatment increased methane production by 50% (155 L CH_4 kg^{-1} VS) compared with untreated algae (103 L CH_4 kg^{-1} VS) during the reactor run in the AVRs. After post-digestion, the ultimate methane yields for pretreated algae were 23–27% higher than for the untreated control (Figure 11b).

A higher methane yield (about 25%) and VS removal were achieved with AVRs operated at 37ºC than with comparable CSTRs with the same average OLRs. The results show that AVRs fed with microalgae can be operated at 20°C and even down to 16°C, but below this temperature, the methane production decreased markedly. Zhang et al. (2012), in their study on artificial wastewater in fixed-bed bioreactors, found that 17°C and 15°C were the temperature thresholds where COD removal and methane production rapidly decreased, which is in agreement with the results in the present study. AD at temperatures close to 20°C is carried out by acclimatized mesophilic microorganisms capable of living at lower temperatures with reduced activity (Kashyap et al. 2003). An adaptation period at temperatures below 20ºC or the use of true psychrophilic microorganisms (Kashyap et al. 2003) could allow digestion of microalgae at temperatures <15ºC, as shown by Heubeck and Craggs (2010) with piggery waste.

Since effluent is not continuously removed from AVRs (as opposed to CSTRs), the AVR volume would need to be approximately four times that of a CSTR to treat the same amount of algae biomass. However, the loading parameters were not optimized in this study, and a smaller volume may be feasible. The longer SRT in AVRs should reduce the need for post-digestion or sludge stabilization that is common with CSTRs, both of which require additional storage capacity. Another drawbacks from digestion at low temperatures may be the inefficient pathogen removal when wastewater is used and loss of dissolved methane with digestate. Even AD at 35ºC is not necessarily efficient for pathogen removal (Borowski et al. 2014), so a further disinfection step may be needed for any agricultural use of digestate. As methane is more soluble at colder temperature, more methane is found from the digestate at low temperature AD. Methane is a strong greenhouse gas, and recovering the soluble methane may require attention.

The results of the present study show that AVRs require approximately double the SRT at 20ºC to achieve a methane yield similar to that at 37ºC. These results agree with earlier findings on AD temperature with different substrates (Kashyap et al. 2003). Hydrolysis, especially protein hydrolysis, may have been the rate-limiting step of AD at 20ºC, as indicated by the low VFA concentrations during experiment and high protein content in the residual solids (Table 16). Both freeze-thaw and low-temperature retreatments enhanced methane yield and VS removal in AVRs at 20ºC. Higher methane production of freeze-thaw pretreated algae was also found in CSTRs at 37ºC. The ultimate methane yield from AD at 20ºC increased with pretreatment of the algae biomass, although the yields were still slightly lower than those from untreated algae at 37ºC. The elevated mineralization of phosphorus and nitrogen in AVRs with pretreated feeds further indicate improved degradation (Table 16).

Table 16. The characteristics of digestates from reactor trials with wastewater microalgae (**II**).

	AVRs 20ºC	AVRs 37ºC	Untreated	3.8 h, 50–57°C	Freeze-thaw	Ambient	CSTRs
Liquids (fill 1)							
TS (%)	0.37 (0.01)	0.30 (0.01)	0.10 (0.01)	0.13 (0.01)	0.12 (0.01)	0.10 (0.01)	1.33 (0.01)
VS (%)	0.16 (0.01)	0.10 (0.01)	0.02 (0.01)	0.03 (0.01)	0.03 (0.01)	0.03 (0.01)	0.91 (0.01)
VS/TS (%)	43 (1)	34 (1)	24 (2)	26 (1)	27 (2)	28 (6)	68 (1)
SCOD (g L^{-1})	1.6 (0.2)	0.5 (0.1)	0.35	0.74	0.75	0.31	0.45 (0.1)
TVFA (g L^{-1})	0.7 (0.1)	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Liquids from total mass (%)	41	45	49 (46–50)	48 (47–49)	55 (1)	46 (1)	n.a.
Liquids (fill 2)							
TS (%)	0.25 (0.02)	0.25 (0.03)	n.a.	n.a.	n.a.	n.a.	1.37 (0.03)
VS (%)	0.07 (0.01)	0.10 (0.02)	n.a.	n.a.	n.a.	n.a.	0.93 (0.02)
VS/TS (%)	26 (1)	38 (3)	n.a.	n.a.	n.a.	n.a.	68 (1)
SCOD (g L^{-1})	0.9 (0.1)	1.1 (0.2)	n.a.	n.a.	n.a.	n.a.	0.5 (0.1)
TVFA (g L^{-1})	0.22 (0.02)	<0.01	n.a.	n.a.	n.a.	n.a.	<0.02
Liquids from total mass (%)	28–29	33 (1)	n.a.	n.a.	n.a.	n.a.	n.a.
TKN mg L^{-1}	690 (90)	870 (110)	410 (50)[1]	710 (90)[1]	750 (90)[1]	380 (50)[1]	1090 (20)
NH_4 mg L^{-1}	780 (70)	950 (80)	460 (40)[1]	720 (60)[1]	650 (60)[1]	390 (40)[1]	500 (70)
Total P mg L^{-1}	170 (30)	150 (30)	130 (20)[1]	180 (30)[1]	170 (30)[1]	100 (20)[1]	180 (30)
Dissolved P mg L^{-1}	180 (20)	140 (20)	140 (20)[1]	250 (20)[1]	250 (20)[1]	140 (20)[1]	80 (30)
Solids (post-digestion)							
TS (%)	2.87 (0.05)	2.91 (0.04)	2.78 (0.10)	2.69 (0.06)	2.99 (0.06)	3.02 (0.16)	n.a.
VS (%)	1.87 (0.02)	1.87 (0.04)	1.95 (0.07)	1.84 (0.04)	2.03 (0.04)	2.19 (0.12)	n.a.
VS/TS (%)	65 (1)	64 (1)	70 (1)	68 (1)	68 (1)	73 (2)	n.a.
Protein (% TS)	35	31	40	38	35	43	n.a.
Fat (% TS)	7	7	10	9	12	10	n.a.
Carbohydrate (% TS)	17	24	19	21	21	19	n.a.
Ash (% TS)	35	36	20	32	32	28	n.a.
Solids from total mass (%)	71–72	67 (1)	51 (50–54)	52 (51–53)	45 (1)	54 (2)	n.a.
TKN mg L^{-1}	2400 (320)	2300 (290)	1900 (230)	1880 (230)	2400 (300)	2200 (270)	n.a.
Total P mg L^{-1}	230 (40)	250 (40)	230 (40)	240 (40)	210 (30)	250 (40)	n.a.

n.a. = not applicable, [1]Nutrient concentrations are after the first fill in experiment 2

7.2.3 Pulp and paper biosludge

Pulp and paper biosludge was studied in long-term (400 d) reactor trials with three parallel CSTRs. An original biosludge with a low TS of 1.2% was used to feed reactors during the first 60 days, after which the TS of the biosludge was increased to 2.5–4.3% with gravity settling to allow an increase in the OLR. One of the parallel reactors was fed with thermally pretreated (121ºC) biosludge during days 313–400. The HRT was decreased from 20 d to 14 d and finally to 10 d.

At the beginning of the trial, when the reactors were fed with unsettled biosludge that had a low solid content (TS 1.2%), the methane yield (46–98 L CH_4 kg^{-1} VS, averaging 71 L CH_4 kg^{-1} VS) and in particular the methane concentration of the biogas fluctuated widely (50–62%, Figure 10c). After the introduction of settled biosludge, the weekly methane yields from seven different biosludge batches in reactor trials differed by a maximum of 19% from the averages (74 and 77 L CH_4 kg^{-1} VS) with HRTs of 20 and 14 d. This result strongly demonstrates that while the biosludge collected at varying times over the course of the year did differ, especially in TS and nutrient concentrations, the degradability and methane yield remained the same. Shortening the HRT from 20 d to 14 d did not affect the methane yield, and the sCOD (<0.6 g L^{-1}) and the VFA (<0.1 g L^{-1} as sCOD) concentrations also remained at a low level (Table 17).

The methane yield increased immediately after the introduction of thermally pretreated biosludge (Figure 10c). With an HRT of 14 d, the methane yield (138 L CH_4 kg^{-1} VS) from thermally pretreated biosludge was 75% higher than from untreated biosludge (79 L CH_4 kg^{-1} VS). When the HRT was shortened to 10 d (OLR 2.2 kg VS m^{-3} d^{-1}), the methane yield from pretreated biosludge averaged 134 L CH_4 kg^{-1} VS, nearly the same as the yield from the previous HRT of 14 d. However, the methane production from untreated biosludge ceased immediately after the HRT was shortened to 10 d (Figure 10c). After the introduction of pretreated sludge, TS removal increased from 6% to 17% and the sCOD concentration of the digestate increased from 0.5 g L^{-1} to 1.4 g L^{-1}. When the HRT was decreased to 10 d, the sCOD in the digestate further increased to 1.6 g L^{-1}, while the VFA concentration remained <0.1 g L^{-1} (Table 17). The increase of sCOD with pretreated biosludge digestate is in contrast to that of untreated biosludge, where sCOD concentrations decreased notably (from 0.6–0.3 g L^{-1}) and the VFA concentration remained <0.1 g L^{-1} as sCOD after the HRT was shortened to 10 d, indicating the failure of the hydrolysis step of AD.

Biosludge lacks soluble nutrients, as the NH_4 concentration was only <81 mg L^{-1} and the PO_4 concentration <20 mg L^{-1}. With AD, nitrogen in the solid fraction of biosludge in particular was mineralized. When untreated biosludge was digested, an NH_4 concentration 170–590 mg L^{-1} and a PO_4 concentration of 12–18 mg L^{-1} (N:P from 10 to 49) was measured from digestate. The highest NH_4 concentration (590 mg L^{-1}) was from the period when nitrogen-rich (81 g kg^{-1} TS) biosludge was used as a substrate. Nutrient mineralization was enhanced after pretreatments, when NH_4 concentration in digestate was 486–496 mg L^{-1} and PO_4 concentration 28–33 mg L^{-1} (N:P from 15 to 18). The optimal N:P ratio for *Chlorella* cultivation is found to be 7, clearly lower than in pulp and paper digestate. For example, *Scenedesmus* sp. is reported to require more nitrogen and an N:P ratio of 30 (Cai et al. 2013). Fouilland et al. (2014) found that *Scenedesmus* sp. can grow in higher NH_4 concentration than

Nannochloropsis and *Dunaliella*, and high growth rates were achieved with NH_4 concentrations >100 mg L^{-1}. The N:P ratio of pulp and paper biosludge digestate is likely suitable for the cultivation of *Scenedesmus* sp., and this was supported by findings in this work (IV), as *Scenedesmus* sp. was the dominating species when mixed culture native species were cultivated in pulp and paper digestate (25% digestate, 75% water). The growth of *Scenedesmus* in pulp and paper digestate this work (IV) was limited, biomass concentration being maximum 0.22 g L^{-1}. However, it must be noted that the growth was not optimized, and in our recent studies, exceptionally high growth (biomass concentrations up to 9 g L^{-1}) of *Scenedesmus* in 100% pulp and paper digestate have been achieved (unpublished data).

Among the challenges in pulp and paper biosludge digestate for algae cultivation may be the widely variable nutrient concentrations. In addition, when pretreatment was applied the color of liquid phase of digestate darkens, potentially limiting light availability.

Table 17. Operation parameters, methane yields, methane concentrations, and digestate characteristics during 400 d reactor trials with pulp and paper biosludge (**III**). When applicable, standard deviations are enclosed in parentheses.

	Biosludge				Pretreated biosludge (at 121°C)	
Operation parameters						
Time (d)	1–64	65–301	302–364	365–400	313–364	365–400
OLR (kg VS m^{-3} d^{-1})	0.5	1.0–1.6	1.6–1.9	1.8–2.2	1.6–1.9	1.8–2.2
HRT (d)	20	20	14	10	14	10
Gas production						
Methane yield (L CH_4 kg^{-1} VS)	70 (16)[a]	76 (8)[d]	78 (3)[f]	0	138 (11)[h]	134 (13)[i]
Methane yield (m^3 CH_4 t^{-1} ww)	0.6 (0.2)[a]	2.0 (0.5)[d]	2.1 (0.2)[f]	0	3.0 (0.5)[h]	2.5 (0.3)[i]
Methane conc. (%)	56 (6)[a]	63 (3)[d]	62 (2)[f]	59 (4)[f]	63 (2)[h]	63 (2)[i]
TS removal (%)	3[a]	6–6	6–7	n.m	17	13
VS removal (%)	9[a]	9–10	9–10	n.m	25	19
Digestate characteristics						
VFA (mg L^{-1} as sCOD)	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
SCOD (g L^{-1})	0.7 (0.1)[b]	0.6 (0.1)[e]	0.6 (0.1)[g]	0.4 (0.1)[c]	1.4 (0.1)[c]	1.6 (0.1)[c]
TKN (g L^{-1})	n.m.	1.3–1.8	1.7 (0.1)[c]	1.4 (0.1)[c]	1.5 (0.1)[c]	1.6 (0.1)[c]
TKN (g kg^{-1} TS)	170 (20)[c]	44–65	54	n.m.	58 (6)[c]	59 (6)[c]
NH_4-N (mg L^{-1})	170 (20)[c]	210–590	300–370	n.m.	496 (25)[c]	486 (24)[c]
Total P (mg L^{-1})	n.m.	126–235	n.m.	n.m.	n.m.	n.m.
Total P (g kg^{-1} TS)	n.m.	6.7–8.0	n.m.	n.m.	n.m.	n.m.
PO_4-P (mg L^{-1})	n.m.	n.m.	12 (2)[c]	18 (2)[c]	28 (3)[c]	33 (4)[c]
pH	7.0–7.2	6.9–7.2	7.2	7.0–7.1	7.0–7.2	7.1–7.2
Lignin (% TS)	n.m.	46	n.m.	n.m.	n.m.	n.m.
Carbohydrates (% TS)	n.m.	7	n.m.	n.m.	n.m.	n.m.

n.m. = not measured. Number of replicates: [a]32; [b]16; [c]3; [d]75; [e]40; [f]14; [g]8; [h]6; [i]4
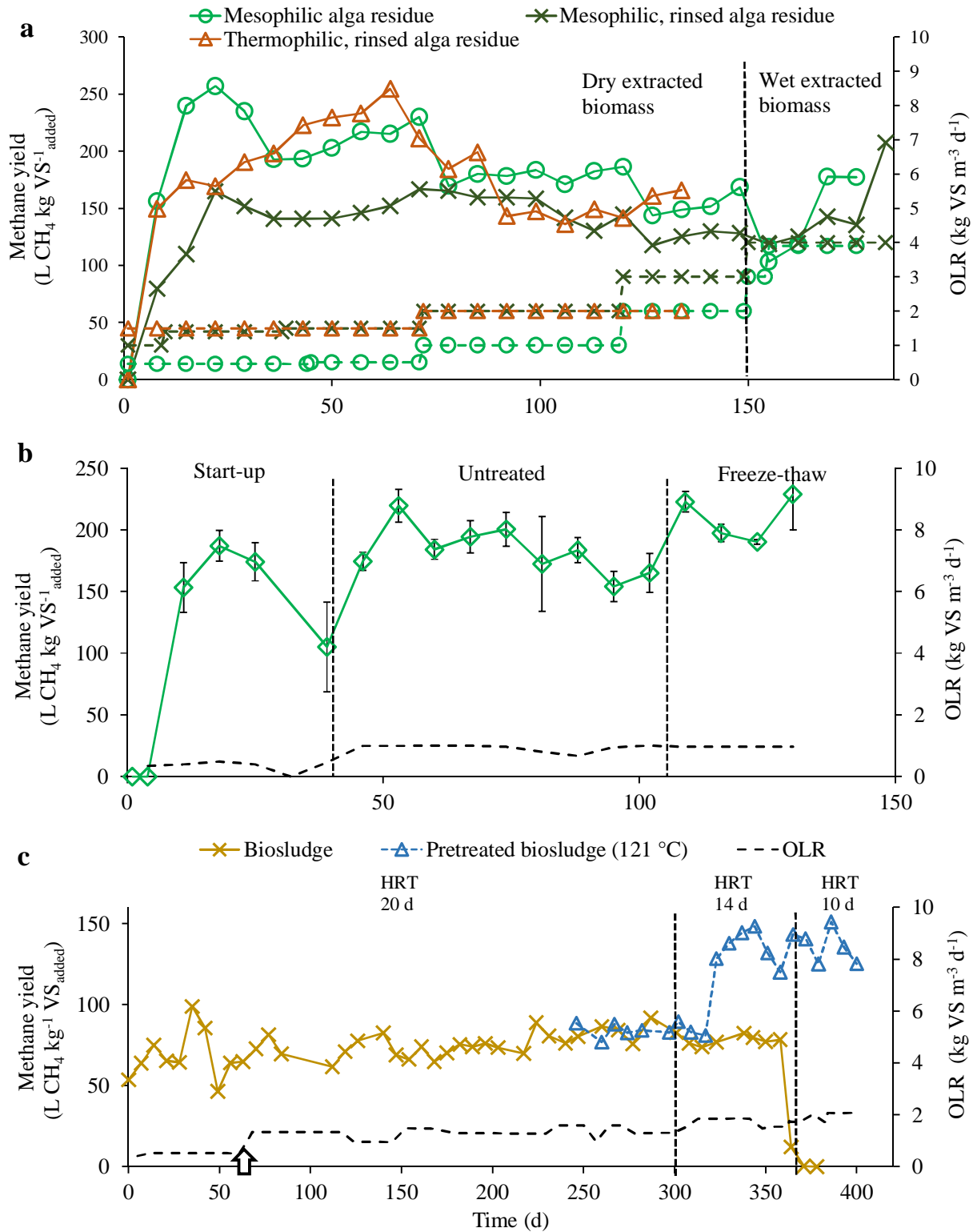
Figure 10. Methane production in continuous reactor trials with CSTRs; **a:** microalgae residues; **b:** wastewater microalgae; and **c**: Pulp and paper biosludge (arrow marks the start of feeding with settled biosludge.
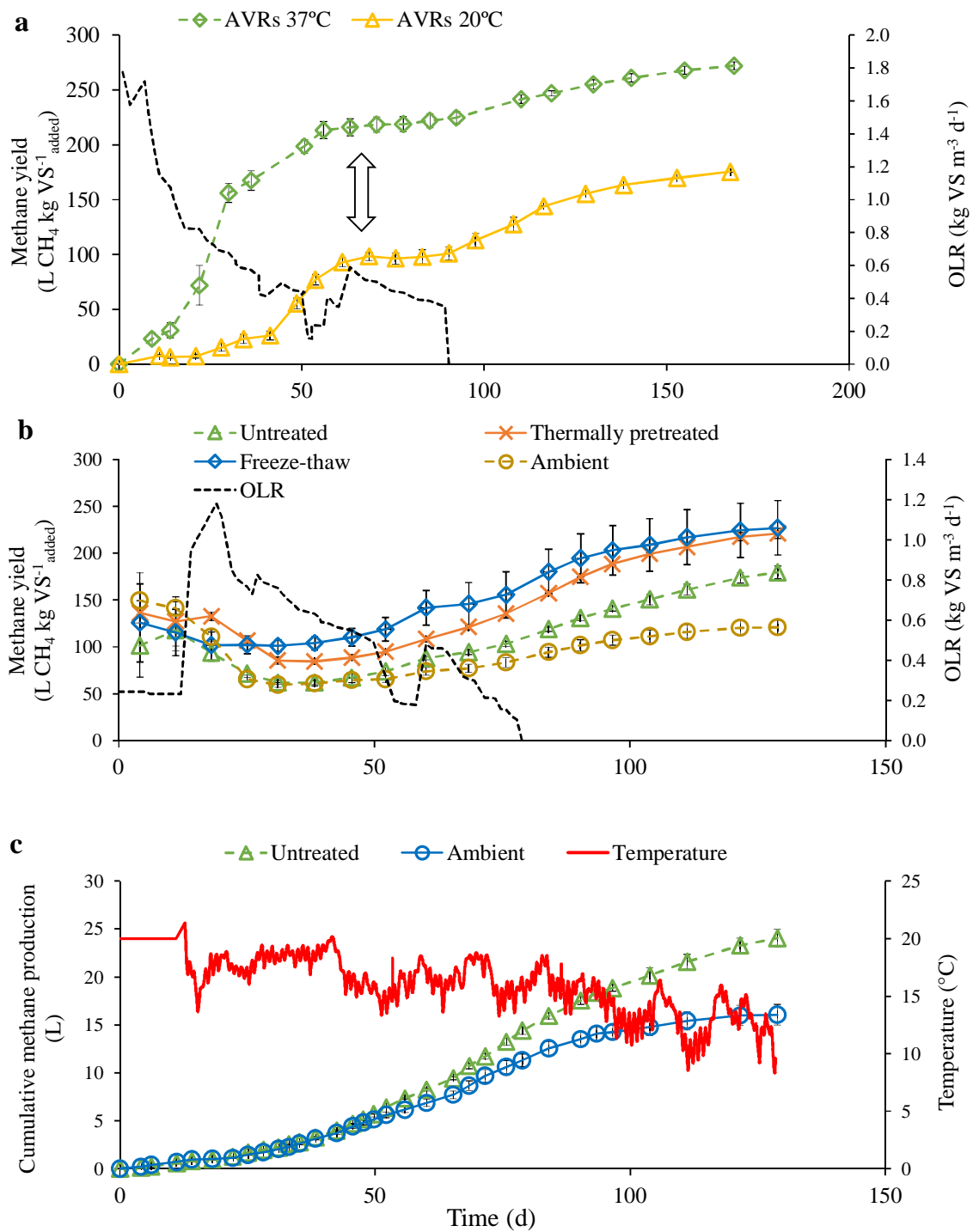
Figure 11. Methane production in continuous reactor trials with AVRs. **a:** Wastewater-grown microalgae digested at 20°C and 35°C, double arrow shows the emptying of liquid phase; **b:** wastewater-grown microalgae digested at 20°C with low-temperature and freeze-thaw pretreatments, and at ambient temperature without pretreatments; **c:** AVR at 20°C and ambient temperature.

7.2.4 Energy balance assessment

The energy balances for AD and thermal pretreatments of microalgal biomass and pulp and paper biosludge were calculated using results obtained in this thesis and are given in Tables 18 and 19. For microalgal biomass, the energy balance was calculated for one-hectare cultivation in wastewater HRAP, and for pulp and paper biosludge for production of 3500 t TS^{-1} a^{-1} the value is given in a range of typical sludge production (2900–4000 t TS^{-1} a^{-1}) from one pulp and paper mill (Stoica et al. 2009).

The microalgal biomass productivity of 5.9 g VSS m^{-2} a^{-1} was used in the calculations; this is the yearly average production achieved recently in pilot-scale wastewater HRAPs in Hamilton, New Zealand (Mehrabadi et al. 2016). The same HRAP was used to produce algal biomass in Paper II in this study. When 5.9 g VSS m^{-2} a^{-1} productivity and a solid concentration of TS 2%, often achieved with simple gravity settling, was used in calculations, the AD energy balances for algal biomass were only positive ($\Delta E > 0$, $E_o/E_i > 1$) with digestion in AVRs that possessed heating that kept the temperature above 16ºC (heating only when the temperature drops below this). Despite higher methane production when using mesophilic (35ºC) CSTR for digestion, AD consumed more energy than it produced, mainly due to the increased heating requirement. Pretreatment decreased the energy balance and energy ratio and also made them negative in AD using AVRs. The energy balance for untreated in AVR was 30 MWh a^{-1}, but here the energy consumed for algae cultivation was not included. By assuming an energy consumption of 0.34 W m^{-2} (Chen et al. 2015) to run HRAP, algae cultivation would consume 30 MWh a^{-1}, meaning that the system would not be feasible for energy production purposes. The primary energy production potential (via AD) from one hectare with 5.9 g VSS m^{-2} a^{-1} productivity (E_o) 39–57 MWh a^{-1} is about the same level as that reported for maize 33–89 MWh a^{-1}, but as maize has a high TS content (about 20–30%), the feeding volume is substantially lower and therefore AD requires less energy for heating.

Energy balances were calculated for algal biomass with two different solid concentrations. While a TS of 2% is commonly achieved with gravity settling, a comparative TS concentration of 4% would likely require an additional dewatering step. However, because higher biomass concentration would reduce the total volume of feed, the heat demand for pretreatment and reactor heating as well as the reactor size would be reduced. Indeed, increasing algal biomass concentration (TS 4%) also turned the energy ratio of AD in CSTR slightly positive (E_o/E_i 1.57) when the energy cost of additional dewatering was not included. However, the highest achieved energy balance (33 MWh a^{-1}) in AVRs is still rather low when the energy input for algae cultivation is taken into consideration. Pretreatments also had a negative effect on the energy balance even when it was calculated for TS 4% biomass.

Table 18. Energy balance assessment for AD of microalgal biomass from one-hectare cultivation. Balances are calculated for three algal biomass productivities (5.9, 10 and 20 $gVSS\ m^{-2}\ d^{-1}$) and TS concentrations of 2 and 4% of harvested biomass.

	CSTR 35°C						AVR 16°C			
	TS 2%			TS 4%			TS 2%		TS 4%	
	Untreated	60°C	80°C	untreated	60°C	80°C	untreated	60°C	untreated	60°C
5.9 gVSS m^{-2} d^{-1}										
$E_{i,heat}$ (MWh a^{-1})	51	74	85	29	42	49	8	53	5	16
$E_{i,electricity}$ (MWh a^{-1})	3	3	3	2	2	2	0.5	0.5	0.5	0.5
E_o (MWh a^{-1})	47	54	57	47	54	57	39	48	39	48
ΔE (MWh a^{-1})	**-6.6**	**-23.0**	**-31.7**	**17.2**	**9.9**	**6.1**	**30.2**	**-5.5**	**33.6**	**32.0**
E_o/E_i	**0.88**	**0.70**	**0.64**	**1.57**	**1.22**	**1.12**	**4.55**	**0.90**	**7.44**	**2.94**
10 gVSS m^{-2} d^{-1}										
$E_{i,heat}$ (MWh a^{-1})	41	51	59	25	31	34	12	55	7	32
$E_{i,electricity}$ (MWh a^{-1})	5	5	5	3	3	3	1	1	0.5	0.5
E_o (MWh a^{-1})	80	91	96	80	91	96	66	82	66	82
ΔE (MWh a^{-1})	**33.7**	**34.3**	**32.0**	**52.5**	**58.0**	**59.3**	**52.7**	**25.8**	**58.2**	**49.2**
E_o/E_i	**1.72**	**1.60**	**1.50**	**2.89**	**2.74**	**2.60**	**5.05**	**1.46**	**8.80**	**2.49**
20 gVSS m^{-2} d^{-1}										
$E_{i,heat}$ (MWh a^{-1})	68	88	102	41	51	59	17	96	12	55
$E_{i,electricity}$ (MWh a^{-1})	11	11	11	5	5	5	2	2	1	1
E_o (MWh a^{-1})	161	183	193	161	183	193	131	164	131	164
ΔE (MWh a^{-1})	**82**	**84**	**80**	**114**	**126**	**128**	**113**	**67**	**118**	**108**
E_o/E_i	**2.03**	**1.85**	**1.70**	**3.44**	**3.20**	**3.00**	**7.07**	**1.68**	**10.10**	**2.91**

To improve algal energy production per cultivation area, it is clear that areal biomass productivity has an important role (Sutherland et al. 2015). Annual harvestable algal biomass productivity of 9.2 g VSS m^{-2} a^{-1} has been reported for the same pilot HRAPs where algae recycling was used (Park et al. 2013). Here, the calculation was done using 10 g VSS m^{-2} a^{-1} productivity. With the harvested biomass concentration of TS 2%, the energy balance is positive for all options (33.7 MWh a^{-1} in CSTR and 52.7 MWh a^{-1} in AVR). If an algal concentration of TS 4% can be used, the energy balance in CSTRs increases to the same level as AVRs due to reduced heat demand and higher methane production.

To estimate the highest possible potential for algae AD, reported (Park et al. 2013) peak productivity (20 g VSS m^{-2} a^{-1}) from the same HRAPs was used as an optimistic option, although this is neither the harvestable value nor constant throughout the whole year. With this, the highest energy balance of 128 MWh a^{-1} would be gained with AD in CSTR after pretreatment at 80ºC.

The results here agree with earlier studies, where hydrothermal and thermal pretreatments have been found to decrease the energy ratio for low solids algal biomass (reviewed by Passos et al. 2014a). However, Passos and Ferrer (2014) have reported also contradictory results, and calculated that a pretreatment of algal biomass at 75ºC and 95ºC would improve the energy ratio, primarily due to higher achieved improvements in methane yields (70% improvement up to 310 L CH_4 kg^{-1} VS) compared to this work. The energy ratios for AD (algae cultivation not included) in their study were comparable (E_o/E_i 0.52–1.27) to those of this study with CSTRs. Although pretreatments here not necessarily seem directly favorable from an energy standpoint, they improve methane yield, and they can notably increase energy production if excess heat that would otherwise be wasted can be used.

For pulp and paper biosludge, the energy balance was calculated with the average original TS in this study (1.3% (III)) and the TS content after an additional settling step (4%). In addition, different biosludge temperatures were used; 13.8ºC is the average yearly temperature in Hamilton, New Zealand and is used to compare the AD of microalgae and biosludge at the same location. However, the wastewater temperature from a pulp and paper mill can be notably higher (e.g., 38–70ºC in Suvilampi et al. (2003)), and the activated-sludge process has also been demonstrated in thermophilic temperatures (55ºC).

If the sludge temperature is 13.8ºC, the energy balance and energy ratio (E_o/E_i 0.15–0.91) are both clearly negative with all calculated options. If a biosludge temperature of 35ºC is assumed, energy balances turn positive except for the pretreatment of TS 1.3% biosludge. Increasing sludge concentration clearly improves energy balance, and although pretreatments decrease the energy ratio compared to untreated sludge, a pretreatment at 121ºC improves the energy balance. The highest energy balance of 1.8 GWh a^{-1} (3500 t TS^{-1} a^{-1}) would be achieved with AD in CSTR after pretreatment at 121ºC and an HRT of 10 d.

HRAP area required to treat liquid fraction of digestate from AD of pulp and paper biosludge with an average sludge production is 7.5 ha, when calculated based on original biosludge TS concentration of 1.3%, TS removal of 10% in AD (without pretreatment), solid fraction TS

content of 25% after digestate dewatering, HRAP (0.3 m depth) retention time of 8 d and dilution ratio of 1:4 (digestate:water). If TS concentration of raw biosludge is 4%, required HRAP area would decrease to 2.2 ha, about two times the area than HRAP used to treat effluent from AD of wastewater solids in Chistchurch, New Zealand (Craggs et al. 2015).

Table 19. Energy balance assessment for AD of pulp and paper biosludge from one average size mill. Balances are calculated for two different temperatures (13.8 and 35ºC) and TS concentrations (1.3 and 4%) of raw biosludge.

| | TS 1.3%, HRT 14 d | | TS 4%, HRT 14 d | | TS 4%, HRT 10 d |
	Untreated	121ºC	Untreated	121ºC	121ºC
Biosludge temperature 13.8ºC					
$E_{i,heat}$ (MWh a^{-1})	7215	11291	2430	3761	3706
$E_{i,electricity}$ (MWh a^{-1})	449	449	146	146	117
E_o (MWh a^{-1})	1764	1764	1940	3478	3478
ΔE (MWh a^{-1})	**-5900**	**-9976**	**-636**	**-429**	**-345**
E_o/E_i	**0.23**	**0.15**	**0.75**	**0.89**	**0.91**
Biosludge temperature 35ºC					
$E_{i,heat}$ (MWh a^{-1})	578	4655	273	1604	1549
$E_{i,electricity}$ (MWh a^{-1})	449	449	146	146	117
E_o (MWh a^{-1})	1764	1764	1940	3478	3478
ΔE (MWh a^{-1})	**737**	**–3339**	**1521**	**1728**	**1812**
E_o/E_i	**1.72**	**0.35**	**4.6**	**2.0**	**2.1**

Conclusions

This work examined AD of microalgae under psychrophilic, mesophilic, and thermophilic conditions. The results showed that pretreatments and reactor designs could improve AD of microalgal biomass but that case-specific energy balance assessments are needed to ensure the feasibility of algal AD.

In addition to low harvestable algal productivity, relatively low methane production from microalgae was the main reason for the weak energy balances of algal AD. The BMPs for wastewater-cultivated algae varied between 154 and 273 L CH_4 kg^{-1} VS (**Papers II and IV**), which was less than 50% of the calculated theoretical methane production (**Paper II**). These results suggest that pretreatments at temperatures of 60–80ºC, potentially available as spare heat, or freeze-thaw pretreatment could improve the BMPs by 11–27%, with the highest production achieved herein being 292 L CH_4 kg^{-1} VS (**Papers II and IV)**

Another challenge in AD of algal biomass is the low solid concentration of wastewater microalgae biomass. In this work, the TS of algae harvested by gravity settling from a pilot-scale HRAP was 1.7–1.8%, which was low for conventional AD in a CSTR. In this work, the energy balances in unmixed AVRs, operated at low digestion temperatures (ambient, 16–20°C) (**Paper II**), were better than those of mesophilic CSTRs with a low solid concentration ($< 4\%$) of algal biomass. In AVRs, solid and hydraulic retention times can be decoupled, allowing longer degradation times. Consequently, AVRs provided 25% higher methane yields than conventional CSTRs at 37°C. In an ambient temperature (Hamilton, New Zealand), methane yields were 37–66% of the yields achieved at conventional mesophilic digestion temperatures ($\sim$37°C), but methane production ceased when the temperature dropped below 15°C.

In this work, AD of biomass residue, lipid-extracted marine microalga (*Nannochloropsis* sp.) cultivated for renewable diesel production, was successfully demonstrated in laboratory-scale CSTRs (**Paper I**). The study showed that methane production from algae residue was higher in thermophilic than in mesophilic processes. However, thermophilic AD was also more vulnerable to ammonia inhibition at low OLRs (2 kg VS m^{-3} d^{-1}). Ammonia inhibition originated from the high nitrogen content (65–70 g kg^{-1} TS) of the microalgae. These findings suggest that mesophilic processes are better than thermophilic ones when microalgae biomass is used as the sole substrate in AD. The results also showed that, unlike ammonia, the salt content of marine-cultivated microalgae did not affect AD. However, the salt content in digestate may limit its use as a fertilizer.

Recent research has shown that algae biofuels may only be feasible when multiple products are produced and the by-products are utilized in a biorefinery concept. In this work, AD of pulp and paper industry biosludge, currently considered waste, was used to produce methane and solubilize nutrients for algae cultivation. This work (**Paper III**) demonstrated the long-term mesophilic AD of biosludge, with a HRT of 14 d. The characteristics of the biosludge, especially its nutrient concentration, varied markedly over time, possibly affecting downstream algae cultivation. The methane yield from the biosludge was low (77 L CH_4 kg^{-1} VS) but was enhanced by 77% (138 L CH_4 kg^{-1} VS) by a thermal pretreatment step at 121°C. Pretreatment

also resulted in AD with a shorter HRT of 10 d, enabling a smaller reactor size and decreasing energy consumption. However, the overall energy balance and feasibility of the thermal pretreatment, as well as the separation of the liquid fraction from the digestate for algae cultivation, require further research.

This work, along with other recent research, showed that, despite quite low methane yields, AD of microalgae is technologically mature, and the major challenges of algal biofuels lie in increasing the cost-efficiency and reliability of microalgal cultivation and harvesting steps. Unless harvestable algal productivities can be improved, achieving a positive energy balance in AD of microalgae seems uncertain. Therefore, integrating algae cultivation into other industries, such as in the recovery of nutrients from waste streams and the production of value-added products, could pave the way toward full-scale applications.

Recommendations for future research

Most recent research, including this thesis, has focused on green algae species, many of which have a tough cell wall that is resistant to degradation. Blue-green algae (cyanobacteria) offer many of the same benefits as green algae but do not have a rigid cell wall. Therefore, more research could focus on blue-green algae species.

In this thesis, wet-extracted algae residues showed much higher methane production compared with dry-extracted residues. The present experiments were unable to shed light on this finding. However, it suggests that the impact of extraction method on methane production should be further studied if algae biomass is intended for use in diesel production or lipids extracted for other purposes.

The potential of the AVR, which was the focus of the present work, for low-solid substrates could be further investigated and optimized. The use of a sieve structure, which would separate the solid and liquid phases, could further enhance the settling of solids in the reactor, further increasing the SRT and reducing the required reactor volume.

Further research is also required to assess the possibility of using algae to recover nutrients from specific types of waste streams, such as digestate with a high cadmium content from AD of pulp and paper biosludge. The pretreatments applied to biosludge prior to AD affect the characteristics of the digestate and may influence algae growth. For instance, according to the results of this thesis, digestate containing not only more nutrients but also a darker colored liquid fraction is typical after pretreatments. If cadmium is present in the growth medium, it is also important to investigate its fate: For instance, can algae take up cadmium, or will it remain in the effluent after algae cultivation? Investigating the fate of cadmium in the digestate (i.e., whether it is found in the solid or liquid fraction) is also important for AD of pulp and paper sludge, as the cadmium may affect downstream processing of the digestate.

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ORIGINAL PAPERS

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MESOPHILIC AND THERMOPHILIC ANAEROBIC LABORATORY-SCALE DIGESTION OF NANNOCHLOROPSIS MICROALGA RESIDUES

by

Kinnunen, V., Koskinen, P. & Rintala, J., 2014
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Mesophilic and thermophilic anaerobic laboratory-scale digestion of *Nannochloropsis* microalga residues

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HIGHLIGHTS

- Methane production from *Nannochloropsis* microalga residue was studied.
- Wet extracted alga had superior biomethane potential compared with dry extracted.
- Anaerobic digestion at 55 °C was more efficient compared with digestion at 35 °C.
- Thermophilic process was inhibited because of ammonia with low loading.
- Salt from marine alga did not inhibit anaerobic digestion.

ARTICLE INFO

ABSTRACT

This paper studies methane production using a marine microalga, *Nannochloropsis* sp. residue from biodiesel production. Residue cake from *Nannochloropsis*, oils wet-extracted, had a methane potential of 482 L CH_4 kg^{-1} volatile solids (VS) in batch assays. However, when dry-extracted, the methane potential of residue cake was only 194 L CH_4 kg^{-1} VS. In semi-continuous reactor trials with dry-extracted residue cake, a thermophilic reactor produced 48% higher methane yield (220 L CH_4 kg^{-1} VS) than a mesophilic reactor (149 L CH_4 kg^{-1} VS). The thermophilic reactor was apparently inhibited due to ammonia with organic loading rate (OLR) of 2 kg VS m^{-3} d^{-1} (hydraulic retention time (HRT) 46 d), whereas the mesophilic reactor performed with OLR of 3 kg VS m^{-3} d^{-1} (HRT 30 d). Algal salt content did not inhibit digestion. Additional methane (18–33% of primary digester yield) was produced during 100 d post-digestion.

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1. Introduction

Currently, vegetable oils, waste oils, and animal fat wastes are used as raw materials for production of bio-based diesel fuels. The biofuel industry seeks sustainable, new, non-food feedstocks. In recent years, microalgae have often been noted as a potential feedstock to produce biofuels. Prior to refining renewable diesel from algae, oil must be extracted from the algal cells. This is typically performed using solvents from dried or wet algal biomass, the algal residue representing most of the total biomass. Utilizing this by-product (referred to as residue cake), in an environmentally sound way is essential to the sustainability and profitability of algal biodiesel production (Chisti, 2007; Sialve et al., 2009). Anaerobic digestion (AD) is one means of recovering energy and nutrients from the residue cake. In addition to produced methane gas, nutrients in the substrate are retained in the AD digestate, which can be further refined as fertilizers or circulated back to algae cultivation. Recently, catalytic hydrothermal gasification (CHG) has shown another promising concept to produce fuel gas and recover nitrogen and phosphorus from algal residues (Frank et al., 2013). The advantages of AD are relatively simple and mature technology and no need for high process temperature or pressure. The major challenges of AD are fugitive methane emissions, relatively long treatment time and possible instability of the AD process. Indeed, decreased methane yield or even the failure of AD process may arise from inhibition, which is caused by excess ammonia, high salt concentration, or difficult degradability of algae (Yen and Brune, 2007; Sialve et al., 2009).

An optimal substrate C/N ratio for AD of algae is suggested to range from 12 to 30 (Yen and Brune, 2007; Ehimen et al., 2011);

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lower values would make the process susceptible to ammonia inhibition. For algae, much lower C/N ratios of 5.3–10.2 (Elser et al., 2000; Yen and Brune, 2007; Ehimen et al., 2009) and 4.4–5.6 (Ehimen et al., 2009; Park and Li, 2012) have been reported for fresh biomass and residue cakes, respectively.

Avoiding fresh water consumption may be crucial for algal biofuel sustainability (Pate et al., 2011). When cultivated in marine water, algal biomass and also algal residues contain sea salt, which is known to have an inhibitive effect on AD (Lefebvre et al., 2007; Chen et al., 2008). Indeed, salt concentration of marine-cultivated *Dunaliella tertiolecta* has been found to inhibit AD (Lakaniemi et al., 2011). However AD has been demonstrated with marine alga *Tetraselmis* and saline wastewaters (Asinari Di San Marzano et al., 1982; Lefebvre et al., 2007). Not only affecting on AD process, salt could also limit the use of digestate as a plant fertilizer. As low sodium concentration as ~500 mg L^{-1} in digested municipal solid waste can be phytotoxic (McLachlan et al., 2004). For comparison, the sodium concentration of sea water is approximately 11 000 mg L^{-1}.

Numerous studies on AD of algae have been completed, but experimental investigations into methane production from algal residues are scarce. Methane production from algae in continuous or semi-continuous reactors has been studied using an organic loading rate (OLR) between 0.01 (De Schamphelaire and Verstraete, 2009) and 6 kg volatile solids (VS) m^{-3} d^{-1} (Yen and Brune, 2007; Park and Li, 2012). The OLRs at the high end of the range were reported to lead to overloading of the process (Yen and Brune, 2007; Ehimen et al., 2009; Park and Li, 2012). Hydraulic retention time (HRT) is an important factor, since algae degrade slowly. Based on the literature and experiments with semi-continuous completely stirred tank reactors (CSTR), Ras et al. (2011) found that methane yield from algae increased with increasing HRT, up to an HRT of 30 days. Beyond this HRT, the methane yield did not increase, meaning either that these algae are not further degradable or that degradation needs considerably more time. AD of algae in a thermophilic process has been reported to produce higher methane yields than in mesophilic digestion (Golueke et al., 1957; Zamalloa et al., 2012). However, this may be species-related, as noted by Zamalloa et al. (2012), who found no difference in biogas yields between thermophilic and mesophilic processes when digesting marine alga *Phaeodactylum tricornutum*, but about 24% greater biogas yield from freshwater alga *Scenedesmus obliquus* in a thermophilic process.

Ehimen et al. (2009, 2011) studied methane production potentials and digestion of fresh water *Chlorella* residue after oils were extracted, using batch assays and semi-continuously fed reactors. These authors concluded that HRT and OLR are significant variables affecting methane production. They also found that some solvents, like the chloroform used for extraction, may inhibit the anaerobic process. Park and Li (2012) reported that co-digestion of *Nannochloropsis salina* residues with grease waste increased both methane yield and allowed higher OLR compared to digestion of these substrates alone. Recently, Keymer et al. (2013) found that the oil extraction process itself can increase methane yield from *Scenedesmus*-dominated algal biomass.

The objective of this study was to evaluate the feasibility of a semi-continuous AD process in recovering energy, in the form of methane, from a marine green microalga, *Nannochloropsis* sp. residue cake. Two *Nannochloropsis* residue cakes were tested in biomethane potential batch assays, one dry-extracted (referred to as N1) and the other wet-extracted (referred to as N2). Dry-extracted residue cake (N1) alone was further studied in mesophilic and thermophilic CSTRs. OLRs were increased and HRTs decreased stepwise during the 5-month reactor trial to determine feasible loading potentials and to monitor the role of ammonia and/or salt inhibition.

2. Methods

2.1. Feeds and inocula

Nannochloropsis sp. residue cake was used after extraction of oils as a feed in AD. The algal biomass was cultivated in open marine water ponds (at Israel), mixed constantly with paddle wheels. Algal biomass may contain small traces of other algae species. Fig. 1 shows a flow chart of materials and processes used in this study. Methane production potential was determined in batch assays from two residue cakes, oils extracted by dry (N1) or wet extraction (N2), but only N1 was used in semi-continuous reactor trials. Part of both N1 and N2 biomasses was rinsed to reduce the salt concentration.

2.1.1. Oil extraction and rinsing

Extraction of oils from air-spray dried algal biomass (N1) was performed via methanol and hexane extraction. Algal biomass was first treated with methanol (10 kg MeOH per 1 kg algal biomass as dry matter) in a column, and methanol was circulated through it with six separate solvent batches (600 L h^{-1} – 1 h each rinse) at room temperature. After this, biomass was treated 4 times with technical grade hexane (6 kg hexane per 1 kg algal biomass as dry matter) at room temperature. Then, biomass was separated by filtration and solvent was removed under a hood and finally with vacuum over distillation in laboratory at temperature less than 100 °C. In wet extraction (N2), ethanol was first added to wet algal biomass (15% dry matter content, 23 kg EtOH per 1 kg algal biomass as dry matter) and part of the ethanol and water was removed by overdistilling in a pressurized reactor under 3 bar pressure at 80 °C (10 kg EtOH per 1 kg algal biomass as dry matter). Next, technical hexane was added (12 kg hexane per 1 kg algal biomass as dry matter) and the mixture was cooked for 1.5 h at 75 °C temperature in 3 bar pressure. Biomass was separated by filtration and rinsed two times with technical-grade hexane and solvent was removed under a hood. Materials were stored in closed bags in a dry environment, at 22 °C during the experiments.

To reduce sea salt concentration, part of the residue cake was rinsed with water. Algal biomass was mixed with 10 times its volume of 22 ± 1 °C distilled water, in a 250 mL decanter, mixing for 30 min with magnetic stirrer at 200 rpm. Subsequently, the algal residue was separated by centrifuge for 5 min at 3000 rpm, and the rinsing procedure was repeated.

For reactor experiments, residue cake was diluted with distilled water to a total solids (TS) content of 10% and was used as feed. Stocks of algal feeds were prepared once per week and stored at 4 ± 1 °C.

2.1.2. Inocula

Inocula used were digestate from a mesophilic sewage sludge digester at the municipal wastewater treatment plant in Jyväskylä, Finland and from a thermophilic digester, digesting sewage sludge and biowaste in Mustasaari, Finland. Mesophilic inoculum was collected three times within a 6-month interval to obtain fresh inoculum for each methane production potential assay and reactor experiment. Inocula were stored at 4 °C before use, and for 1 week before experiments inocula were kept in open canisters at 35 °C (mesophilic) and 55 °C (thermophilic) to activate methanogenic micro-organisms and to reduce residual degradable material.

2.2. Methane production potential and post-digestion assays

Methane production potentials of substrates were determined in triplicate batch assays using 119 mL glass bottles at 35 °C.
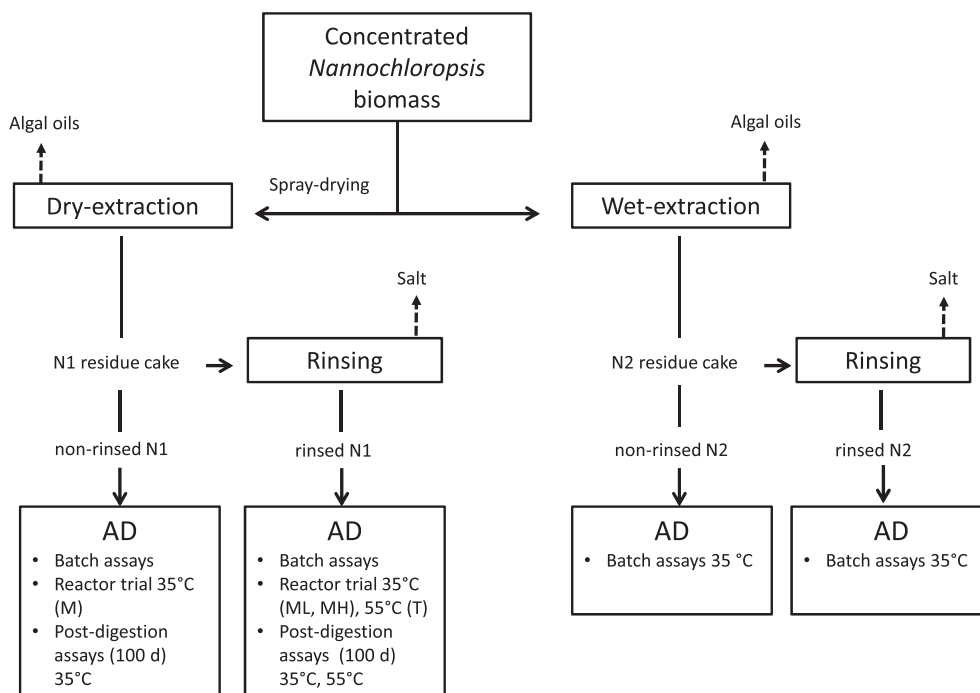
Fig. 1. A flow chart illustrating the overall experimental set up. To remove algal oils, wet or dry extraction was conducted. Rinsing was done to reduce the salt concentration of algal residues. N1 = *Nannochloropsis* residue, air-dried prior to extraction, N2 = *Nannochloropsis* residue, wet-extracted. M = reactor fed with N1 operated at 35 °C, ML = reactor fed with rinsed N1 feed operated at 35 °C with lower OLRs, MH = reactor fed with rinsed N1 and operated at 35 °C with higher OLRs, and T = reactor fed with rinsed N1 feed operated at 55 °C.

Inoculum (30 mL) and substrate were added into bottles, using a $VS_{substrate}: VS_{inoculum}$ ratio of 1:1. Distilled water was added to make a total liquid volume of 60 mL, and $4\,g\,L^{-1}$ $NaHCO_3$ was added as buffer. Inoculum alone with distilled water was assayed as control (three replicates) and its produced methane subtracted from that of the substrates. Bottles were flushed for 1 min with nitrogen gas and sealed gas tight with rubber caps and aluminium seals. Methane concentration of produced gas was measured 1–4 times per week. Bottles were manually shaken prior to measurement.

Residual methane potentials of semi-continuous reactor digestates were determined in duplicate or triplicate post-digestion assays in 58 or 119 mL serum bottles, similarly to methane production potentials. Particular volumes (10 or 30 mL) of reactor digestate were added and bottles were incubated either at 35 or 55 °C according to reactor set-up.

2.3. Reactor experiments

Four CSTR reactors, each of size 5 L and liquid volume of 4 L were used. The CSTRs were constructed of glass and fitted with outlets at the top of the reactor for biogas collection, feeding, and digestate withdrawal. Biogas was collected into aluminium foil bags Tesseraux (TECOBAG) via gastight tubes (Masterflex Tygon). Reactors were stirred continuously with magnetic stirrers at 350 rpm.

Reactors were inoculated with 4 L of inocula, and feeding was started after 1 week's incubation (referred to as day 0). CSTRs were run for a maximum of 150 days, and fed every weekday (Monday to Friday) with N1 residue cake. One reactor was fed with non-rinsed residue cake and operated at 35 °C (labelled as M). Three reactors were fed with rinsed residue cake. Two of these reactors were operated at 35 °C with different OLRs (ML; lower OLRs 0.5–1.0 kg VS m^{-3} d^{-1} and MH; higher OLRs 1.5–3.0 kg VS m^{-3} d^{-1}), while the third reactor was operated at 55 °C (T) (Fig. 1). The experimental

objective was to gradually increase the OLRs (and shorten HRTs) to determine the loading potential of each process.

2.4. Analysis and calculations

To measure organic matter and nitrogen contents of feeds and digestates, TS and VS, soluble (SCOD) and total (TCOD) chemical oxygen demand, NH_4^+ and total kjeldahl nitrogen (TKN) were analysed as described in Bayr et al. (2012). NH_3, the un-ionised fraction of NH_4^+ and the most inhibitive for AD process, was calculated using dissociation constants. To follow pH in reactors, Radiometer pHM82 standard and Mettler Toledo SevenEasy pH meters were used. To measure oil content of algal residues, fatty acid content was determined with GC-FID according to EN ISO1530:2002 standard. The volume of the biogas produced was measured using a water displacement method. The methane content of the biogas and concentration of volatile fatty acids (VFA), intermediate products of AD process, were measured with GC-FID as described in Bayr et al. (2012). To measure algae salt concentration, sodium analyses from substrates were conducted with ICP-MS instrument. VFA, SCOD, and NH_4^+ were determined from filtrate, first centrifuged with Sanyo Harrier 18/80 centrifuge at 5500 rpm and then filtrated through GF/A glass microfiber filter.

Methane production was converted and is given using norm conditions (NTP): temperature 273 K and pressure 1 000 mbar. Residual methane potentials are given as total cumulative methane production per added VS (into the reactor) and digestate volume after 30 and 100 d digestion. In reactor experiments, methane yields and VS removals were calculated using averages from the three final weeks of each OLR. The lost proportion of VS during rinsing was calculated as a ratio of mass loss of VS in rinsing and original mass of VS used in rinsing. OLR and HRT were calculated based on the daily feed volumes and feed VS contents in semi-continuous operation; OLRs for continuous operation are 5/7 times OLR reported in this study, because the reactors were fed 5 days/week.

Table 1
Characteristics of *Nannochloropsis* residue cakes. Characteristics of *Nannochloropsis* residue cakes N1 and N2. Standard deviations are shown in parentheses. N1 residue was dry-extracted and N2 residue wet-extracted, including pressure treatments. Methane production potentials are obtained from batch assays. Reactor feed was diluted to TS 10% with distilled water.

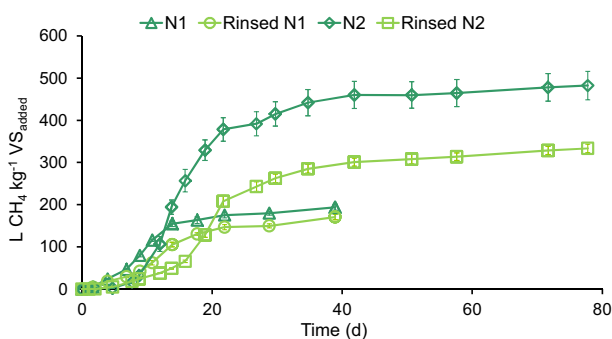|	Original residue cake		Diluted feeds used in CSTRs	
	N1	N2	N1	Rinsed N1
TS (%)	91.2 (0.3)	80.3 (0.1)	10 (1)	10 (1)
VS (%)	66.4 (2.4)	66.3 (0.5)	7.3 (0.1)	9.1 (0.1)
VS/TS (%)	73 (3)	83 (1)	73 (3)	91 (1)
pH	6.6	6.5	6.5	6.8
TKN (mg g^{-1} TS)	70 (2)	n.d.	n.d.	n.d.
Na (g L^{-1})	n.d.	n.d.	2.9	0.4
Oil content (% of TS)	15	6.3	n.d.	n.d.
Biomethane potential (L CH$_4$ kg^{-1} VS)	194 (8)	482 (34)	194 (8)	171 (7)

Fig. 2. Cumulative methane production of algal residues during batch assays. Methane production of algal residues during batch assays. N1 = *Nannochloropsis* residue, air-dried prior to extraction, N2 = *Nannochloropsis* residue, wet-extracted.

3. Results and discussion

3.1. Characteristics of Nannochloropsis residue cake and the effects of rinsing

Nannochloropsis residue cakes (N1 and N2) were characterised prior to and following rinsing. The aim was to study the effects of salt concentration on methane yield using marine-cultivated alga. Oil was extracted from both biomass samples by use of solvents; but unlike for N1, the extraction step for N2 included treatments at 3 bar pressure (75 and 80 °C) and was conducted for wet biomass. N1 residue cake contained 15% oils from dry matter and N2 contained 6%. Rinsing reduced the sodium concentration of N1 by 85% (Table 1). In addition, on average, 7 ± 4% of VS was lost when air-dried residue cake (N1) was rinsed. For N2, the lost portion averaged 17 ± 10%.

Methane potential was 148% higher for the N2 residue cake (482 L CH$_4$ kg^{-1} VS) than for N1 residue cake (194 L CH$_4$ kg^{-1} VS), despite the higher remaining oil content of N1 (Fig. 2, Table 1). This implies that the extraction method may have an effect on algal residue degradability. Unlike dry extraction, the wet extraction included thermal treatment under pressure. The treatment physically disrupts the biomass, likely enabling the protein and carbohydrate components of the residue cake to be more available for anaerobic micro-organisms. Also, the presence of solvents could affect methane production potential, e.g. chloroform in chloroform/methanol-extracted biomass has been shown to inhibit the anaerobic process (Ehimen et al., 2009). In the present study, methanol was used for extraction of N1 and ethanol for N2, but the presence of solvents or inhibitors in assayed biomass is not likely, as residue cakes were dried after extraction, allowing solvents to evaporate. Further, rinsing would have decreased ethanol/methanol concentrations. Rinsing decreased the methane potential (per VS) of residue cakes by 31% for N2 and 12% for N1. The decreased methane potential after rinsing indicates that the VS lost with rinsing water was more degradable than the retained VS.

The results in the present study support the findings of Keymer et al. (2013), that oil extraction (biomass dried at 55 °C) affects the methane production potential of algae. From the results obtained from batch assays, these authors reported oil extraction of *Scenedesmus* biomass to increase the methane potential by 33% compared to raw alga (from 180 to 240 L CH$_4$ kg^{-1} VS). Further, a 110% increase in methane production potential (380 L CH$_4$ kg^{-1}-VS) was achieved when oil extraction was followed by high-pressure thermal treatment at 170 °C and 8 bar pressure (Keymer et al., 2013). Ehimen et al. (2009) reported a methane production potential of 230–280 L CH$_4$ kg^{-1} VS for fresh water alga *Chlorella* sp. residues and approximately 440 L CH$_4$ kg^{-1} VS for non-extracted biomass. In the Ehimen et al. (2009, 2010) study, extraction of *Chlorella* was conducted on algal biomass first dried at 80 °C. In the present study, the methane potential of N1 residue cake (194 L CH$_4$ kg^{-1} VS) was lower than that reported for *Chlorella* (Ehimen et al., 2009) and *Scenedesmus* (Keymer et al., 2013) residues earlier. However, the methane potential of the wet-extracted residue cake, N2 (482 L CH$_4$ kg^{-1} VS), was higher than earlier found for algal residues (Table 2). This suggests that oil extraction may increase methane production potential of algae as reported by Keymer et al. (2013) (the present study does not include a comparison between extracted and non-extracted biomass). Alternatively, air-drying prior to the extraction step of N1 may make algal cells harder, hindering biomass degradability under the studied anaerobic conditions. Dried algal biomass has been shown to produce lower methane potential than the same biomass digested on a wet basis (Asinari Di San Marzano et al., 1982; Mussgnug et al., 2010) (Table 2). Despite lower methane production potential, N1 was tested in reactor experiments in this study, and further research for wet-extracted residue cake is needed.

3.2. Methane yield, VS removal, and loading during reactor experiments

The OLRs and process performances are presented in Figs. 2 and 3 and are summarized in Table 3. When the OLR was increased and the HRT shortened, the methane yield showed a slightly decreasing trend in all reactors. The highest tested OLRs showed undisturbed performance in terms of methane yield and VFA concentration in mesophilic reactors (3 kg VS m^{-3} d^{-1} (HRT 30 d) for rinsed residue cake (MH), and 2 kg VS m^{-3} d^{-1} (HRT 36 d) for non-rinsed residue cake (M)). With these OLRs, the methane yields were 128 L CH$_4$-kg^{-1} VS for rinsed residue cake (MH) and 156 L CH$_4$ kg^{-1} VS for non-rinsed residue cake (M). Methane yields for non-rinsed residue cake were 5–16% higher than yields for rinsed residue cake with the same OLRs (Table 3).

Table 2
Methane production of algal biomasses. Methane production of various algal biomasses in batch and continuous experiments.

Experimental set-up	Substrate	T (°C)	OLR (kg VS m^{-3} d^{-1})	HRT (d)	Methane yield (L CH$_4$ kg^{-1} VS)	References
Batch	*Nannochloropsis* 1 (dried, oil extracted, Na$^+$ 2.9 g L^{-1})	35	n.a.	39	194	This study
Batch	*Nannochloropsis* 2 (wet extracted)	35	n.a.	77	482	This study
Batch	*Chlorella* spp. (dried, froze)	37	n.a	37	443[a]	Ehimen et al. (2009)
Batch	*Chlorella* spp. (dried, froze, oil extracted)	37	n.a	35	234–283[a]	Ehimen et al. (2009)
Batch	*Scenedesmus* spp.	38	n.a.	35	180	Keymer et al. (2013)
Batch	*Scenedesmus* spp. (dried, oil extracted)	38	n.a.	35	240	Keymer et al. (2013)
Batch	(*Scenedesmus* spp.) (dried, oil extracted, HPTH[b])	38	n.a.	35	380	Keymer et al. (2013)
Batch	*Dunaliella tertiolecta* (Na$^+$ 2.1 g L^{-1})	37	n.a.	49	24	Lakaniemi et al. (2011)
Semi-continuous, 4 L	*Nannochloropsis* 1 (dried, oil extracted, Na$^+$ 2.9 g L^{-1})	35	2	36	156	This study
Semi-continuous, 4 L	Rinsed *Nannochloropsis* 1 (dried, oil extracted)	35	3	30	128	This study
Semi-continuous, 4 L	Rinsed *Nannochloropsis* 1 (dried, oil extracted)	55	1.5	61	220	This study
Semi-continuous, 1 L	*Nannochloropsis salina* (wet extracted)	37	2	40	130	Park and Li (2012)
Continuous, 1.5 L	*Chlorella* spp. (dried, froze, oil extracted)	35	n.a.	15	245	Ehimen et al. (2011)
Semi-continuous, 1 L	*Chlorella vulgaris*	35	0.7[c]	28	240	Ras et al. (2011)
Semi-continuous, 4 L	*Scenedesmus* spp. and Chlorella spp.	35	2	10	90	Yen and Brune (2007)
Semi-continuous, 4 L	*Scenedesmus* spp. and Chlorella spp.	35	4	10	143	Yen and Brune (2007)
Semi-continuous, 4 L	*Scenedesmus* spp. and Chlorella spp.	35	6	10	136	Yen and Brune (2007)
Continuous, 11 L	*Scenedesmus* spp. and Chlorella spp.	35	1.4	30	268	Golueke et al. (1957)
Continuous, 11 L	*Scenedesmus* spp. and *Chlorella* spp.	50	1.4	30	310	Golueke et al. (1957)
Continuous, 2 L	*Scenedesmus obliquus*	33	2.7	2.2	130	Zamalloa et al. (2012)
Continuous, 2 L	*Scenedesmus obliquus*	54	2.8	2.2	140	Zamalloa et al. (2012)
Continuous, 2–5 L	*Tetraselmis*	35	2	14	310	Asinari Di San Marzano et al. (1982)
Continuous, 2–5 L	*Tetraselmis* (dried)	35	2	14	260	Asinari Di San Marzano et al. (1982)
Continuous, 2–5 L	*Tetraselmis* (dried + NaCl 35 g L^{-1})	35	2	14	250	Asinari Di San Marzano et al. (1982)

[a] Estimated from figure, converted from given methane production per dry weight to methane production per VS using VS/TS share of 94.6%.
[b] HPTH = high pressure thermal hydrolysis (170 °C, 8 bar).
[c] Calculated from data given in L CH$_4$ gCOD^{-1} using COD/VS ratio of 1.5 according Sialve et al. (2009).

In the thermophilic reactor (T), the OLR of 1.5 kg VS m^{-3} d^{-1} (HRT 61 d) provided the only undisturbed performance, yielding 220 L CH$_4$ kg^{-1} VS, 48% more than in the comparative mesophilic reactor with the same OLR and HRT. With the OLR of 2 kg VS m^{-3} d^{-1} (HRT 46 d), methane production and methane concentration of biogas decreased rapidly (Fig. 3, Table 3). Methane yield was still at the same level as in the comparative mesophilic reactor (MH), but the thermophilic one showed clear signs of unstable operation (described in section; pH, VFAs, and COD in reactor experiments). As with methane yields, VS removal also showed a decreasing trend with increasing OLRs. VS removals were 41–42% in all reactors with similar OLR of 2 kg VS m^{-3} d^{-1} (Table 3).

Methane yields in the present study, with similar OLRs, are slightly higher than the 130 L CH$_4$ kg^{-1} VS that Park and Li (2012) reported for wet-extracted *Nannochloropsis* residues in mesophilic process (OLR 2 kg VS m^{-3} d^{-1}, HRT 40 d). These authors' methane yields decreased to almost zero when the OLR was increased from 2 to 3 kg VS m^{-3} d^{-1}. The present results are also within the range of yields obtained in previous studies using fresh algal feeds, where 90–310 L CH$_4$ kg^{-1} VS methane yields were obtained with OLRs of 1–6 kg VS m^{-3} d^{-1} and HRTs of 10–33 d (Table 2). The highest methane yields for non-rinsed (208 L CH$_4$ kg^{-1} VS) and rinsed (220 L CH$_4$ kg^{-1} VS in thermophilic process) N1 residue cakes in reactor experiments with low OLRs (0.5–1.5 kg VS m^{-3} d^{-1}) and long HRTs (61–146 d) were similar to or slightly higher than methane production potentials determined in batch assays (171–194 L CH$_4$). As seen in the results from batch assays, rinsed residue cake produced a lower methane yield than non-rinsed because some of the readily degradable material was lost in the rinsing process.
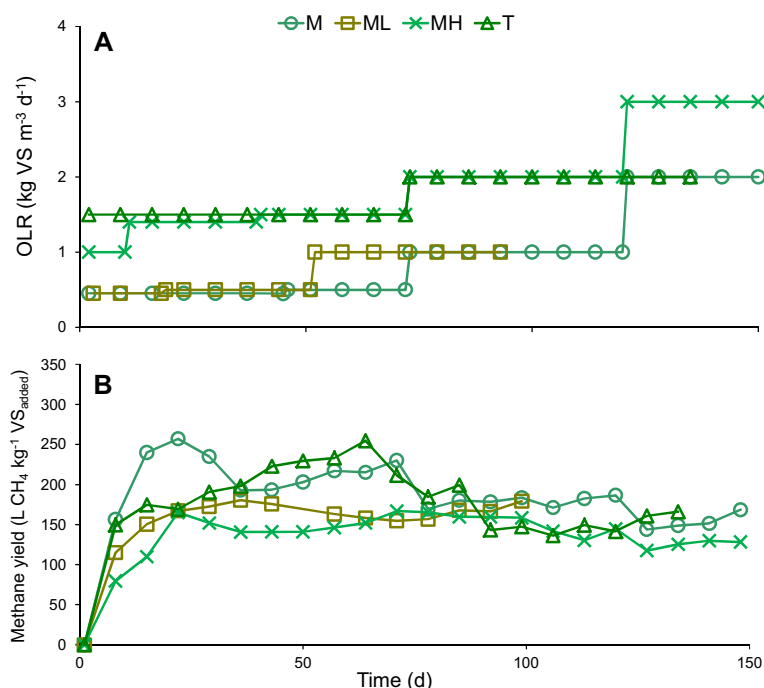
Rinsed N1 residue cake produced 48% more methane in the thermophilic process than in the mesophilic one with the same OLR (1.5 kg VS m^{-3} d^{-1}); this is in accordance with earlier findings (Golueke et al., 1957; Zamalloa et al., 2012). However, the higher productivity in the thermophilic reactor was achieved only during the lowest used OLR; the reactor (T) had unstable operation when OLR was increased. Golueke et al. (1957) reported 40–50% and

50–60% VS removals from algae in mesophilic and thermophilic operation, respectively. This is a higher rate of degradation compared to the present study (33–42%). Algal residues may contain a greater quantity of non-readily degradable organic matter than non-extracted matter; this would explain relatively low VS removals. These results must be taken with caution, as the operational periods with each OLR were only up to 1.2 times the HRT, meaning the whole liquid volume inside the reactor did not change and the reactors did not reach stable operation.

3.3. PH, VFAs and COD in reactor experiments

In all mesophilic reactors, pH remained between 7.2 and 7.5. In the thermophilic reactor, pH increased gradually from the initial 7.5 to 8.0 during the first 50 days, remaining at this level for the rest of the experiment (Fig. 4A). No decrease in pH was seen, even when VFAs began to accumulate in the reactors (Fig. 4C). Higher pH in the thermophilic than mesophilic reactors was probably due to CO_2; it is more soluble at lower temperatures and, as an acid, decreases the pH of mesophilic reactors. Higher temperature also favours the NH_3 form of ammonium, which as a base further increases pH. Indeed, NH_3 concentration increased simultaneously with pH in the thermophilic reactor during the first 50 days (Fig. 2A and C). NH_3 may have also acted as a buffer, keeping pH constant when VFAs began to accumulate (Angelidaki and Ahring, 1994).

VFAs are intermediate products of AD process and VFA accumulation may result from inhibition or overloading of some microbiological step of AD. Total volatile fatty acids (TVFA; total concentration of acetic, propionic, iso-butyric, butyric, iso-valeric, valeric and caproic acids) concentration remained below or about 0.2 g L^{-1} in all mesophilic reactors with OLRs up to 3.0 kg VS m^{-3} d^{-1} (HRTs 30 d). Only in the mesophilic reactor with non-rinsed alga (M) did TVFA concentration peak at 0.83 g L^{-1} with OLR of 2 kg VS m^{-3} d^{-1}, (day 144), but levelling out again to below 0.2 g L^{-1} within 2 weeks' time (Fig. 4C).

Fig. 3. OLRs and methane yields during semi-continuous digestion of *Nannochloropsis* residue cake. (A) OLRs and (B) methane yields (per VS) as weekly averages during semi-continuous reactor runs, where (◊) M-reactor fed with N1 operated at 35 °C, (□) ML-reactor fed with rinsed N1 feed operated at 35 °C with lower OLRs, (✕) MH-reactor fed with rinsed N1 and operated at 35 °C with higher OLRs, and (△) T-reactor fed with rinsed N1 feed operated at 55 °C.

Table 3
Experimental set-up, methane yields, and chemical characteristics of digestates during reactor runs. TVFA, SCOD, NH_4^+, and TKN are concentrations at the end of each OLR, analysed from reactor digestate. Post-digestion potentials of reactor digestates are given after 30 and 100 days' batch-digestion. Standard deviations are in parentheses. When only two parallels were conducted, both results are shown. (M) fed with N1 and operated at 35 °C, (ML) fed with rinsed N1, operated at 35 °C with lower OLRs, (MH) fed with rinsed N1 and operated at 35 °C with higher OLRs, and (T) fed with rinsed N1, operated at 55 °C.

	Reactor									
	M			ML		MH			T	
OLR (kg VS m^{-3} d^{-1})[a]	0.5	1	2	0.5	1	1.5	2	3	1.5	2
HRT (d)[a]	146	82	36	183	91	61	46	30	61	46
Days	1–71	72–119	120–149	1–50	51–98	1–71	72–119	120–149	1–71	72–134
Methane yields										
(L CH$_4$ kg^{-1} VS$_{added}$)	208 (15)	180 (6)	156 (11)	174 (6)	165 (10)	149 (10)	147 (13)	128 (3)	220 (22)	155 (26)
(m^3 CH$_4$ t^{-1} FM)	134 (16)	120 (4)	104 (8)	115 (4)	109 (7)	99 (7)	98 (9)	85 (2)	146 (15)	103 (17)
$\left(\text{m}^3\ \text{CH}_4\ \text{m}^{-3}_{\text{liquid vol}}\right)$	0.07 (0.01)	0.13 (0.01)	0.22 (0.02)	0.06 (0.01)	0.12 (0.01)	0.15 (0.02)	0.21 (0.02)	0.27 (0.01)	0.24 (0.03)	0.23 (0.03)
Methane concentration (%)	59 (2)	59 (2)	60 (4)	62 (6)	60 (2)	61 (2)	61 (2)	61 (1)	54 (3)	51 (3)
VS-removal (%)	69 (2)	56 (3)	42 (3)	76 (3)	64 (3)	57 (3)	41 (2)	33 (2)	58 (2)	41 (1)
Post-digestion potentials										
30 d (L CH$_4$ kg^{-1} VS$_{feed}$)	8 (1)	5 (1)	29 (2)	8–8	n.d.	15–16	20–20	23 (1)	20–21	30 (1)
100 d (L CH$_4$ kg^{-1} VS$_{feed}$)	15 (1)	26 (2)	51 (3)	13–13	n.d.	27–28	36–38	42 (2)	44–46	38 (1)
30 d (m^3 CH$_4$ t^{-1} digestate)	0.6 (0.1)	0.4 (0.1)	2.1 (0.1)	0.7–0.7	n.d.	1.3–1.4	1.8–1.8	2.2 (0.1)	1.8–1.9	2.7 (0.1)
100 d (m^3 CH$_4$ t^{-1} digestate)	1.2 (0.1)	1.9 (0.1)	3.7 (0.2)	1.3–1.3	n.d.	2.4–2.5	3.3–3.4	3.9 (0.2)	4.1–4.3	3.5 (0.1)
Total methane production										
(L CH$_4$ kg^{-1} VS$_{added}$)	223 (16)	206 (8)	207 (14)	187 (6)	n.d.	177 (11)	183 (14)	170 (5)	265 (24)	193 (27)
Digestate characteristics										
TVFA (mg L^{-1})	90	50	830	50	20	40	220	100	420	3750
SCOD (g L^{-1})	0.9 (0.1)	1.6 (0.1)	2.5 (0.1)	1.1 (0.1)	1.5 (0.2)	2.2 (0.1)	4.6 (0.1)	5.4 (0.2)	7.1 (0.1)	15.8 (0.5)
NH_4^+ (g L^{-1})	1.0 (0.1)	1.0 (0.1)	1.1 (0.1)	0.8 (0.1)	1.0 (0.1)	0.9 (0.1)	1.2 (0.1)	1.3 (0.1)	1.7 (0.1)	2.9 (0.1)
TKN (g L^{-1})	3.3 (0.5)	3.2 (0.5)	6.2 (0.2)	2.9 (0.8)	3.2 (0.6)	4.6 (0.2)	5.4 (2.0)	6.2 (0.2)	5.5 (0.3)	8.6 (0.2)
NH_4^+/TKN (%)	30	31	18	28	31	20	22	21	31	33
pH	7.4 (0.2)	7.2 (0.1)	7.2 (0.1)	7.4 (0.1)	7.3 (0.1)	7.4 (0.1)	7.4 (0.1)	7.4 (0.1)	7.7 (0.2)	7.9 (0.1)

[a] Calculated by daily feed volume and VS in semi-continuous digestion, n.d. = not determined.

In the thermophilic reactor (T), TVFA concentration increased when OLR was increased from 1.5 to 2 kg VS m^{-3} d^{-1} on day 72, ending at 3.75 g L^{-1} on day 134 (Fig. 4C). Unlike in mesophilic reactors, propionic acid was the main VFA, peaking at 1.43 g L^{-1} at the end of the experiment.

SCOD remained at about 1.5 g L^{-1} in mesophilic reactors, until the OLR was increased to 2 kg VS m^{-3} d^{-1}, at which time the SCOD of both M and MH reactors started to increase slowly, ending at 2.5 g L^{-1} (M) and 4.6 g L^{-1} (MH) (Fig. 4B). During this period, TVFA concentration remained for the most part below 0.2 g L^{-1} (as SCOD)
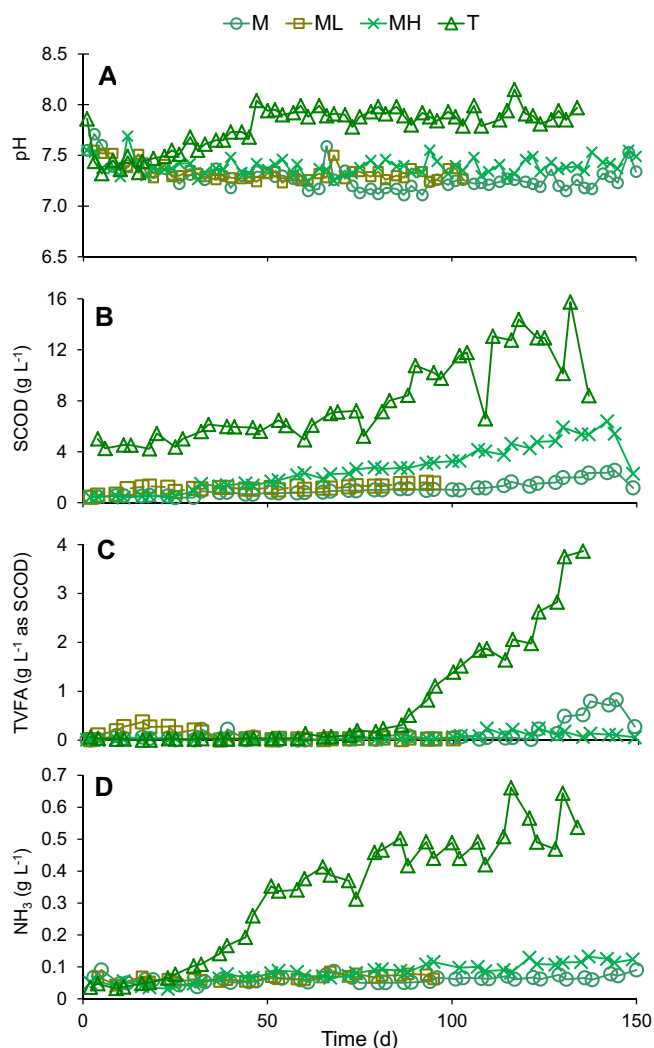
Fig. 4. pH, SCOD-, TVFA-, and NH$_3$ concentrations of digestates during semi-continuous digestion of *Nannochloropsis* residue cake. (A) pH and OLRs (lower data series), (B) SCOD-, (C) TVFA-, (D) NH$_3$-concentrations of reactor digestates during semi-continuous reactor runs where (◇) M-reactor fed with N1 and operated at 35 °C, (□) ML-reactor fed with rinsed N1 feed operated at 35 °C with lower OLRs, (✕) MH-reactor fed with rinsed N1 and operated at 35 °C with higher OLRs, and (△) T-reactor fed with rinsed N1 feed operated at 55 °C.

and therefore does not explain the increase in SCOD. In the thermophilic reactor, the SCOD remained between 4 and 6 g L^{-1}, with OLR of 1.5 kg VS m^{-3} d^{-1}, but peaked at 15.8 g L^{-1} when OLR was increased to 2 kg VS m^{-3} d^{-1}. This was caused mainly by accumulation of VFAs, as about 50% of the SCOD was VFAs (Fig. 4B).

Increasing SCOD concentration in mesophilic reactors with low amounts of TVFA shows accumulation of hydrolysed organic matter in the reactors. This suggests that acidogenesis may have been the limiting step in the AD process, or that this matter was not further degradable under the given conditions. In the latter case, hydrolysis would have been the limiting step, as reported also by Ras et al. (2011) and Zamalloa et al. (2012) for algal feeds. In the thermophilic reactor, acetogenesis was probably the limiting process or was inhibited, as, in addition to acetic acid, intermediate products, propionic acid and isovaleric acid, accumulated.

3.4. Nitrogen solubility in reactor experiments

During the experiments, NH$_4^+$ concentrations increased in all reactors (Table 3). Concentrations were 1.1–1.3 g L^{-1} in mesophilic

reactors with OLRs of 2–3 kg VS m^{-3} d^{-1} (HRTs 30–46 d). In the thermophilic reactor (T), NH$_4^+$ concentration was 1.7 g L^{-1} after OLR of 1.5 kg VS m^{-3} d^{-1} (HRT 61 d) and 2.9 g L^{-1} after OLR of 2 kg VS m^{-3} d^{-1} (HRT 46 d).

NH$_4^+$/TKN ratio of the digestate decreased from 0.28–0.31 to 0.18–0.21 with increasing OLRs in mesophilic reactors (Table 3). In thermophilic reactor (T), NH$_4^+$/TKN ratios were 0.31–0.33.

Nitrogen was not properly mineralized, as only 18–33% of TKN was in the form of NH$_4^+$. In general, the share of NH$_4^+$ decreased with decreasing HRT (Table 3). Ras et al. (2011) showed a similar trend with nitrogen mineralization; 19% under HRT of 16 days, but 68% when HRT was increased to 28 days. Thermophilic reactors showed a higher rate of nitrogen solubilisation than mesophilic reactors, confirming better degradation.

3.5. Ammonia inhibition in reactor experiments

The calculated NH$_3$ concentration remained below 0.2 g L^{-1} in all mesophilic reactors. In the thermophilic reactor (T), NH$_3$ concentration increased, with OLR of 1.5 kg VS m^{-3} d^{-1} up to 0.3–0.4 g L^{-1}, and further to 0.7 g L^{-1} with OLR of 2 kg VS m^{-3} d^{-1} (Fig. 4D).

NH$_3$ concentrations as low as 0.05 g L^{-1} have been found to be inhibitive for AD (Weiland, 1993), but inhibitive concentration can be extremely variable, between 0.1 and 1.1 g L^{-1} (Salminen and Rintala, 2002). The thermophilic AD process is considered to be more sensitive for NH$_3$ inhibition than the mesophilic one, because the NH$_3$/NH$_4^+$ balance favours the NH$_3$ form at higher temperature and pH. This leads to higher NH$_3$ concentration in the thermophilic process than in the mesophilic (Angelidaki and Ahring, 1994). High NH$_3$ levels (0.2–0.7 g L^{-1}) are reported to have an influence, especially on propionic acid degradation in upflow anaerobic sludge bed (UASB) reactors (Calli et al., 2005a,b). Indeed, in the present study, propionic acid was the main component in accumulation of VFAs in the thermophilic reactor (T). This suggests that, most likely, the unstable operation of T-reactor was caused by ammonia inhibition, originating from the high nitrogen content of the residue cake (Table 1).

Since NH$_3$ concentrations were below 0.2 g L^{-1} and acetic acid remained the main component of TVFA throughout the experiment, it is unlikely that the mesophilic reactors were inhibited by ammonia.

Co-digestion of algae with high carbon substrate could improve process stability and prevent ammonium inhibition. Algae digestion with paper cellulose (Yen and Brune, 2007), grease waste (Park and Li, 2012), and glycerine (Ehimen et al., 2009, 2011) has been shown to increase methane yield and reduce inhibition caused by NH$_3$ as the substrate C/N ratio increases.

3.6. Salt inhibition in reactor experiments

Methane yields and operational parameters of the reactor fed with non-rinsed N1 (M) were comparable to yields from reactors fed with rinsed N1 (ML, MH). It may be assumed that inhibition caused by salt concentration in residue cake was insignificant or that the microbial population adapted to increasing salt concentration. Sodium is known to be the inhibitory component of salt in the anaerobic process, since cations can enter microbial cells (Lefebvre et al., 2007). The non-rinsed N1 feed contained only 2.9 g L^{-1} sodium (Table 1), when inhibitory concentrations for sodium are reported to vary from 5.6 to 53 g L^{-1} (Lefebvre et al., 2007; Chen et al., 2008). However, Lakaniemi et al. (2011) found salt inhibition to be the likely reason for low methane production of *D. tertiolecta* marine alga with sodium concentration as low as 2.1 g L^{-1}. Even if it does not affect the AD of marine algal residue, salt may well limit the use of the digestate as a fertilizer.

3.7. Residual methane production from reactor digestate

Residual methane potential (30 and 100 d) of reactor digestates was assayed in post-digestion after each OLR. Residual methane production gradually increased in all mesophilic reactors with increasing OLR and shortening HRT. Therefore, total methane production (reactor + residual methane potential) from each reactor and from 100 days post-digestion of digestate was quite constant after each OLR, 207–223 L CH_4 kg^{-1} VS for non-rinsed N1 and 170–187 L CH_4 kg^{-1} VS for rinsed N1. With the highest OLRs assumed to maintain stable performance, an additional 18% and 33% of methane (calculated per VS added to the CSTR) was obtained during 30 and 100 d post-digestion, respectively (Table 3). Unlike mesophilic reactors, residual methane production from the thermophilic reactor (T) digestate decreased after increased loading, as did total methane production (from 265 to 193 L CH_4 kg^{-1} VS). With an OLR of 1.5 kg VS m^{-3} d^{-1}, an additional 10% and 20% of methane was obtained during 30 and 100 d post-digestion, respectively.

Similar total methane production from reactor trials and 100 d residual digestion after each OLR are in agreement with methane production potentials determined in batch assays. Rinsed residue cake produced a considerably higher total methane yield in the thermophilic process with the lowest given OLR. The yield was 55% higher than the methane production potential in the batch assay, showing more efficient degradation in the thermophilic than mesophilic process. During the first loading in the thermophilic reactor, the inoculum-degradable material, not counted in feed VS, may have increased methane yield, as it was operated only one time in the HRT cycle.

Residual methane potential assays confirm the slow degradability of *Nannochloropsis* residue cake and support the important roles of HRT and OLR in digesting algal residues. Notably, 100-day residual digestion produced 41–122% more methane than 30-day residual digestion, meaning that some organic parts of the alga need more than 100 days to degrade. Ras et al. (2011) speculated that *Chlorella vulgaris* alga is either not further digestible above HRT of 30 days or that degradation needs considerably more time; the present study supports the latter notion, but is not fully comparable, as more concentrated feeds (TS 10%) were used. The limiting steps of digestion are most likely hydrolysis and acidogenesis, as no VFA accumulation occurred. Unlike in all other reactors, post-digestion residual methane production actually decreased from the thermophilic reactor (T) digestate when loading was increased and HRT decreased. This supports the idea that T was inhibited by ammonia, also affecting post-digestion and preventing methanogens from consuming accumulated VFAs. To maximise methane yield and to avoid methane emissions to the atmosphere, covered post-storage is needed for the digestate.

4. Conclusion

Methane production potential obtained from marine algal residue (*Nannochloropsis* sp.), oils extracted, was over a 2-fold from wet-extracted algal residue compared with dry-extracted algal residue. Continuous thermophilic AD process produced 48% more methane than mesophilic process, but was inhibited apparently because of ammonia with low OLR (2 kg VS m^{-3} d^{-1}), while mesophilic process still worked with the highest studied OLR (3 kg VS m^{-3} d^{-1}). The salt concentration of marine algal biomass did not inhibit digestion. Additional methane was produced during the 100-day post-digestion period and must be collected to avoid fugitive methane emissions.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.biortech.2013.12.115.

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II

INFLUENCE OF TEMPERATURE AND PRETREATMENTS ON THE ANAEROBIC DIGESTION OF WASTEWATER GROWN MICROALGAE IN A LABORATORY-SCALE ACCUMULATING-VOLUME REACTOR

by

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Influence of temperature and pretreatments on the anaerobic digestion of wastewater grown microalgae in a laboratory-scale accumulating-volume reactor

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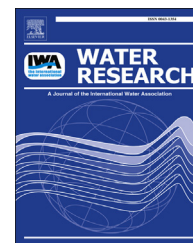
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ABSTRACT

This laboratory-scale study investigated the performance of a low-cost anaerobic digester for microalgae. Low ($\sim$2%) solids content wastewater-grown microalgal biomass (MB) was digested in an unmixed, accumulating-volume reactor (AVR) with solid and liquid separation that enabled a long solids retention time. AVRs (2 or 20 L) were operated at 20 °C, 37 °C or ambient temperature (8–21 °C), and the influence of two pretreatments – low-temperature thermal (50–57 °C) and freeze-thaw – on algal digestion were studied. The highest methane yield from untreated MB was in the 37 °C AVR with 225 L CH_4 kg volatile solids (VS)$^{-1}$, compared with 180 L CH_4 kg VS$^{-1}_{added}$ in a conventional, 37 °C completely stirred tank reactor (CSTR), and 101 L CH_4 kg VS$^{-1}_{added}$ in the 20 °C AVR. Freeze-thaw and low-temperature thermal pretreatments promoted protein hydrolysis and increased methane yields by 32–50% at 20 °C, compared with untreated MB. Pretreatments also increased the mineralisation of nitrogen (41–57%) and phosphorus (76–84%) during digestion. MB digestion at ambient temperature was comparable with digestion at 20 °C, until temperature dropped below 16 °C.

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1.   Introduction

The sustainability of microalgal biofuels has been questioned if chemical fertilizers are needed for cultivation (Lam and Lee, 2012). However, microalgae have been used to assimilate nutrients from wastewaters in high rate algal ponds (HRAP), e.g., in the USA and New Zealand (Craggs et al., 2012). The microalgae could also use CO_2 from the burning of fuels for their growth. Despite recent research, microalgal biofuels remain unviable because of low energy conversion efficiencies. Thus, simplified algal biofuel processes without extensive energy inputs are needed (Lam and Lee, 2012).

Methane production via anaerobic digestion (AD) is a simple way to utilise microalgal biomass (MB) for energy production. However, there are several challenges for the AD of microalgae, including: (1) low solid content of algal biomass, causing unwanted short hydraulic retention time (HRT) in completely stirred tank reactor (CSTR); (2) difficult and slow degradability of algae, leading to low methane yields, compared to theoretical values (Alzate et al., 2012); and (3) high nitrogen concentration of algae, potentially inhibiting the AD process (Sialve et al., 2009).

For CSTR digesters, the low solid concentration of harvested algal biomass, leads to short HRTs (<15 d) and/or low organic loading rates (OLR) (<1.0 g VS L^{-1} d^{-1}). Laboratory AD of microalgae using CSTR, have typically had a HRT of 15–30 days (d) (Ras et al., 2011; González-Fernández et al., 2012a). However, algae have been found to be slowly degradable, and Ras et al. (2011) reported 63% higher methane yield from *Chlorella* at a HRT of 28 d compared with 16 d. Effluent is continuously removed from CSTR digesters, meaning that this digestate contains a portion of recently added and undigested material. Therefore, at HRTs <30 d, CSTR digestate may have significant residual methane potential, which has been found to be mainly (e.g., 88–93%) due to the solid fraction (Angelidaki et al., 2006). Achieving longer solid retention times (SRTs) (>30 d) by e.g., using an unmixed, accumulating-volume reactor (AVR) presented in this study, could offer more complete degradation and higher methane yields for microalgae.

Anaerobic digestion is most commonly conducted at a mesophilic temperature range of 30–40 °C, which requires heating of the reactors in most climate zones. However, with low total solids (TS) substrates such as microalgae, what is actually being heated is mostly water. Digestion in low temperatures (<20 °C), conducted by psychrophilic or acclimatised mesophilic microorganisms, could be a feasible option to decrease energy input (Kashyap et al., 2003). In low temperatures, however, metabolism of microorganisms is reduced; specifically, the hydrolysis step is considered rate limiting (Halalsheh et al., 2011).

To improve algae degradability and thus methane yield without lowering the overall energy balance, there is a need for low-cost pretreatment techniques. Among the pretreatments, low-temperature thermal treatment (<100 °C) could be a sustainable option, since excess heat (50–70 °C) is commonly available, e.g., from combined heat and power units or industrial processes. Another potential low-cost pretreatment could be freeze-thaw of algae biomass, which to our knowledge, has not previously been reported for microalgae, but has been researched for wastewater sludge conditioning (Gao, 2011).

The results of previous studies on low-temperature thermal pretreatment of microalgae have been contradictory. Passos et al. (2013) showed that low-temperature pretreatment for <10 h increased microalgae methane production. Conversely, and Alzate et al. (2012) reported no effect, or even decreased methane production after >12 h pretreatment at 55–60 °C. Indeed, a short treatment time may be favourable for microalgae, for example 4-h at 55–80 °C was found to achieve >80% volatile solids (VS) and chemical oxygen demand (COD) solubilisation (González-Fernández et al., 2012a; Passos et al., 2013). Solubilisation is an important factor for methane production, since hydrolysis is often the limiting step of the AD process.

Freeze-thaw improves wastewater sludge dewaterability and settleability (Örmeci and Vesilind, 2001; Hu et al., 2010; Gao, 2011), and increases COD solubilisation (Gao, 2011) and methane yield (Montusiewicz et al., 2010). As wastewater sludge and microalgal-bacterial biomass share the same unicellular physical properties, freezing could also be considered as a pretreatment method for MB. However, freeze pretreatment may be costly in terms of energy use. In countries with cold winter seasons, nature could provide this energy at a low cost, if suitable freezing infrastructure could be developed.

The objective of this study was to investigate a low-cost AD process for wastewater-grown microalgae. For this purpose, a novel AVR was used and operated at low temperatures. Low-cost pretreatments (low-temperature thermal and freeze-thaw) were also studied to improve algal digestion.

2.   Materials and methods

The experimental set up is shown in Fig. 1. During the nine-month experimental period (June 2011–March 2012), two experiments were conducted. In experiment 1 (five months), MB was digested in AVRs at 20 °C and 37 °C, and as a reference, in a CSTR at 37 °C. In experiment 2 (four months), pretreated (low-temperature thermal and freeze-thaw) MB was digested in AVRs at 20 °C, with untreated MB as the control. During experiment 2, untreated MB was also digested under ambient temperature conditions. The experiments were conducted in duplicate or triplicate.

2.1.   Microalgal biomass and inocula

The HRAP that produced the microalgae used in this experiment were fed with primary settled sewage and were operated with a HRT of 4–8 days, water depth of 0.3 m and horizontal water velocity of 0.15 m s^{-1}. Further details of the HRAP operating conditions are described in Park and Craggs 2010. For reactor feeds, HRAPs were harvested 1–5 times per week over the experimental period, using gravity-settling cones. The TS, VS and pH of each harvest were measured. The dominant species during the nine-month period, as analysed every third week with a microscope, was found to vary over time, consisting of *Pediastrum* sp., *Micractinium* sp. and
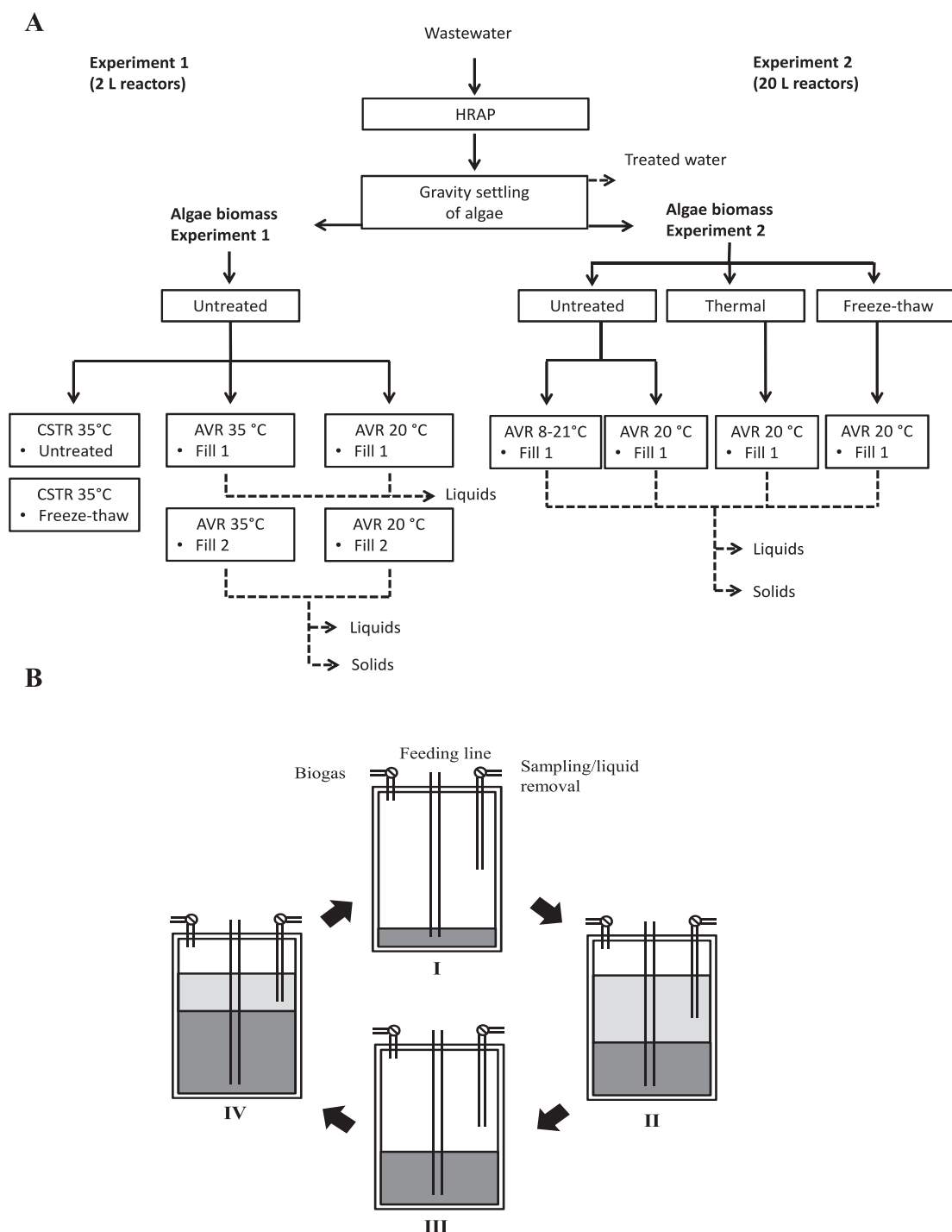
Fig. 1 – Simplified flow chart illustrating the overall experimental set up. (A) Set up of experiments 1 (left) and 2 (right). (B) Schematic diagram of AVR operation. I: fill phase, II: Liquid withdrawal when full, III: Fill phase, IV: Liquid withdrawal when full and solid withdrawal when necessary. The solid and liquid fractions are dark and light grey respectively. The sampling/liquid removal tube is adjustable vertically.

Scenedesmus sp. The comparative reactors were always fed with the same MB harvest.

To characterise lipid, protein, carbohydrate and nutrient content of the harvested MB reactor feed, 200 mL subsamples were sampled approximately every third week during both experiments. These subsamples (5 and 6 respectively) were combined (and stored at 1 ± 1 °C for a maximum period of three months) for further analyses. For all reactors, fresh anaerobic bacterial inoculum originated from an unmixed 5 m³ AVR that digested settled MB at ambient temperature (17–20 °C at the time of collection).

2.2. Pretreatments

Freeze-thaw and low-temperature thermal pretreatment of MB were investigated. For the freeze-thaw pretreatment, the MB was placed in a 1 L plastic bottle and kept at -20 ± 2 °C for 24 h (h), then melted at room temperature (20 ± 2 °C) (referred to as freeze-thaw MB). For the low-temperature thermal pretreatment (referred to as thermally pretreated MB), the MB was incubated in 1-L bottles in an oven (60 ± 3 °C). To test the effect of the incubation time on MB solubilsation, a bottle was removed to room temperature after 2, 4 and 6 h incubation respectively. The temperature was measured with a thermocouple connected to a data logger and the corresponding incubation times when MB temperature was >50 °C were 0.0 h (max. 47 °C), 1.7 h (max. 54 °C) and 3.8 h (max. 57 °C). The 3.8-h pretreatment was chosen for reactor experiments because it provided the best solubilisation. The pretreated MB samples were stored at 1 ± 1 °C for up to 14 d.

2.3. Experimental setup

2.3.1. AVRs
The AVRs were cylinder-shaped glass reactors with total volumes of 2 and 20 L which were unmixed. Each AVR was equipped with an outlet at the top for biogas collection; a tube was attached to the bottom of the reactor for feeding and solid sampling, as well as a vertically adjustable tube below the liquid level for liquid sampling (10 mL once per week) and withdrawing liquids from the reactor. The ambient AVRs were placed outside in a water bath ($\sim$200 L) to moderate diurnal changes in temperature which was monitored using a thermocouple connected to a data logger.

The operation of AVRs is presented in Fig. 1B. AVRs resemble the anaerobic sequencing batch reactors (Massé and Masse, 2000), but without separate reaction and clarification phases and with semi-continuous feeding. The AVRs were started adding 200 mL (experiment 1) or 2000 mL (experiment 2) of inoculum. For experiment 1, the inoculum was initially temperature acclimatised by incubating it for 3 weeks at the reactors' temperatures (20 °C, 37 °C) and subsequently stored for 15 h at 1 °C. Additionally, 3 g L^{-1} NaHCO$_3$ was added as a buffer in experiment 1 on day 0. The AVRs were fed with MB once a day on week days; subsequently, the liquid volume in the reactors increased gradually. The addition of MB was continued until liquid volumes of $\sim$1.8 (for the 2 L AVR, day 64) and $\sim$10.5 L (for the 20 L AVR, day 78) were reached (fill 1). In experiment 1, the liquid fraction was then withdrawn, and feeding was resumed until day 93 (fill 2, days 65–93). Only one fill was monitored during experiment 2. In both experiments, once the liquid volumes were reached and MB addition ceased, measurement of methane production continued (until day 175 in experiment 1 and day 130 in experiment 2), periods referred to as post-MB addition period. At the end of the experiments, the AVRs were emptied, and liquid and solid fractions were weighted and analysed.

The loading parameters during experiments are shown in Table 1. The OLR per liquid volume in the AVRs, decreased (e.g., from 1.7 g VS L^{-1} d^{-1} to 0.3 g VS L^{-1} d^{-1} in Experiment 1) as the liquid volume increased. The average OLR (1.0 g VS L^{-1} d^{-1}) during Experiment 1 was selected to be similar to previously used OLR of CSTR digesters fed with low solids content algae (e.g., Ras et al., 2011). The lower initial OLR in Experiment 2 was used to help minimise the lag phase in methane production that occurred during Experiment 1 (in which the lag phase maybe because of storage of inocula at 1 °C). The SRT was calculated as the average of the time that the solids had accumulated in the reactor, including the post-MB addition period.

2.3.2. CSTRs
The CSTRs used the same 2-L glass reactor jars as the AVRs and were operated with a 1.5-L working volume. The CSTRs were equipped with outlets at the top for biogas collection, feeding, digestate withdrawal and gastight mixing. Reactor contents were stirred with blade stirrers at 200–300 rpm, 15 min on and 15 min off.

The CSTRs were seeded with 1.5 L of inoculum, and acclimatised for one week before starting the experiment (day 0), after which the reactors were fed once a day every weekday. Before adding the MB, 90% of the feeding volume was removed from the reactor content. The OLR was constant at 1.0 g VS L^{-1} d^{-1} (HRT 14–16 d). The reactors were fed with untreated MB

Table 1 – Loading parameters of AVRs and CSTRs. Experiment 1 and 2 for digestion of wastewater-grown microalgae in AVRs and CSTRs. SRTs given are the average times the solids were accommodated in AVRs, including the post-MB addition period. The total length of the experiment is enclosed in parentheses.

MB feed	Temperature (°C)	Reactor	Loading (g VS d^{-1})	OLR[a] (g VS L^{-1} d^{-1})	SRT (d)
Experiment 1					
Untreated	20	2 L AVR (duplicate)	0.45	1.7 →0.3	128 (175)
Untreated	37	2 L AVR (triplicate)	0.45	1.7 →0.3	128 (175)
Untreated[b], freeze-thaw[c]	37	2 L CSTR (triplicate)	1.5	1.0	14–16[d]
Experiment 2					
Untreated	20	20 L AVR (duplicate)	1.0–2.5	1.2→0.3	91 (130)
Thermally pretreated	20	20 L AVR (duplicate)	1.0–2.5	1.2→0.3	91 (130)
Freeze-thaw	20	20 L AVR (triplicate)	1.0–2.5	1.2→0.3	91 (130)
Untreated	Ambient (8–20)	20 L AVR (triplicate)	1.0–2.5	1.2→0.3	91 (130)

[a] In AVRs, the OLR is given at the beginning (highest) and at the end (lowest) of the fill.
[b] Days 0–101.
[c] Days 102–130.
[d] In CSTR SRT = HRT.

during days 0–101 (experiment 1) and freeze-thaw MB during days 102–130 (experiment 2).

2.3.3. Batch assays

Methane potentials for inocula were determined in duplicate batch assays in 1.3 L PET plastic bottles at 20 ± 1 °C and at 37 ± 1 °C. For each bottle, 300 mL of inoculum and distilled water were added to reach a total liquid volume of 600 mL. The bottles were flushed for 3 min with nitrogen gas and closed with gastight caps. The ultimate methane yields of the inocula were subtracted from the methane yields measured in the AVRs.

2.4. Analyses and calculations

Biogas volume was measured either directly using water displacement gas collection cylinders (experiment 1 and batch assays) or by first collecting in gas bags and then measuring volume using water displacement (experiment 2 and CSTRs). Water in the gas-collecting cylinders was acidified with 2 M HCl (pH 2) and salinated with NaCl to minimise gas dissolution. Methane concentrations in the biogas were measured using a portable gas analyser (Geotechnical Instruments, GA2000), calibrated against a standard gas mixture (60% CH_4, 20% CO_2, 20% N_2).

The TS and VS (APHA 2540), COD (APHA 5220 D), total Kjeldahl nitrogen (TKN) (APHA 4000 N_{org} C), total phosphorus (APHA 4500-P E), dissolved phosphorus (APHA 3125 B), ammonical-N (APHA 4500-NH_3), nitrate N + nitrite-N (APHA 4500-NO_3^-) and settleable solids (APHA 2540 F) were analysed according to standard methods. Soluble COD (SCOD), ammonical-N and dissolved phosphorus were measured after filtration through a 0.45-μm membrane filter. Volatile fatty acids (VFAs) were measured by ion chromatography (Hill Laboratories Ltd). pH was measured using a TPS WP-81 pH metre. Protein content was analysed with Leco's total combustion method (AOAC 968.06) and fat with cold extraction using chloroform/methanol (AOAC 969.24). Carbohydrate content was calculated by the differences of protein, fat and ash from TS.

Methane production in each of the AVRs was calculated as the cumulative methane yield (L CH_4) at the end of each fill, and as methane yield per total cumulative VS added into the reactor by the given day of each fill (L CH_4 kg^{-1} VS). In experiment 1 which had two fills, the cumulative methane and VS for both fills were combined. The ultimate methane yield was the methane yield at the end of the experiment, after post-MB addition period. Theoretical methane yields and methane production from VFAs (350 mL g COD^{-1}) were calculated according to the formulas of Angelidaki and Sanders (2004). All gas production results were given under STP conditions (0 °C, 10^5 Pa).

In the CSTRs, methane yield (L CH_4 kg^{-1} VS) was calculated as weekly methane production per weekly VS added. All methane results were calculated as averages of replicate reactors. Methane yields from the CSTRs were calculated as an average of the last three weeks of each experiment.

VS removal in the AVRs was calculated by subtracting the amount of VS in each reactor at the end of the experiment from the total cumulative VS fed into each reactor during the experiment. VS removal in the CSTRs was calculated by subtracting the amount of VS in the digestate during the last week of the experiment from the average VS of the MB fed into the CSTR during the last three weeks of the experiment. The solubilisation degree (S_D) of the MB after pretreatment was calculated as in Alzate et al. (2012).

3. Results

3.1. Algal biomass and effect of pretreatments on biomass properties

The characteristics of the MB (composite samples) that were fed into the reactors are shown in Table 2. The TS and VS of both MB composite samples were 1.7–1.8% and 1.2–1.4%, respectively, while the TS and VS of the individual feed samples during the nine-month experimental period (1–5 samples analysed every week) ranged from 0.8% to 2.5% and from 0.6% to 2.0%, respectively. Low TS and VS concentrations occurred when a poorly settleable algal species (Scenedesmus sp) dominated the HRAP and when algae-eating invertebrate populations were high. Proteins contributed 41–42% of the TS in both feeds, while the ash content of the wastewater grown MB was 26–29%. The ultimate methane yield of the MB used during experiment 1 was 273 L CH_4 kg VS$^{-1}_{added}$ (from AVR experiments at 37 °C after the post-MB addition period), while theoretical methane yields for the MB feeds were 550–563 L CH_4 kg VS$^{-1}_{added}$. The ultimate methane yields per VS removed (462–581 L CH_4 kg VS$^{-1}_{removed}$) are in agreement with theoretical values.

Both pretreatments changed the visual appearance of the MB. Low-temperature treatment changed the biomass colour from green to brown, while freeze-thaw made the biomass aggregate and improved the settleability of the MB (dominated by Pediastrum sp. and Microactinium sp.) (Fig. 2). The duration of low-temperature treatment affected the solubilisation of COD, increasing from 5% after 1.7-h to 11% after 3.7-h treatment.

Table 2 – Characteristics of wastewater-grown algal biomass. The characteristics of MB used as feeds in reactor experiments. Samples are composites of 5 (experiment 1) and 6 (experiment 2) subsamples.

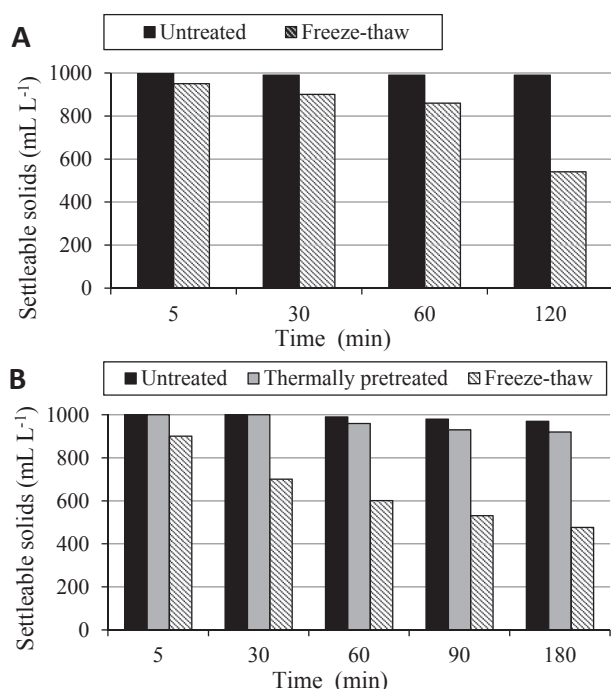|	Experiment 1	Experiment 2
TS %	1.7	1.9
VS %	1.2	1.4
VS/TS %	71	74
pH	6.4	6.5
Protein %TS	41	42
Fat %TS	12	11
Carbohydrates %TS	18	21
Ash %TS	29	26
Theoretical methane yield (L CH_4 kg VS^{-1})	563	550
Ultimate methane yield (L CH_4 kg VS^{-1})	273	n.d.
TKN mg L^{-1}	1100	1100
Total P mg L^{-1}	230	190

n.d. = not determined.

Fig. 2 – **Effects of pretreatments on algae settling. The settleable solids of freeze-thaw, thermally pretreated and untreated (A)** *Pediastrum* **sp.-dominated biomass (2.1% TS, 1.6% VS) and (B)** *Microactinium* **sp.-dominated biomass (1.9% TS, 1.4% VS).**

Table 3 – **Effect of pretreatments on algae COD solubilisation. COD solubilisation of MB after 1.7–3.8 h low-thermal (50–57 °C) and freeze-thaw pretreatments. 2 h (20–47 °C) means MB temperature reached 47 °C during 2 h incubation at 60 °C. S_D means solubilisation degree.**

	TCOD (mg L^{-1})	SCOD (mg L^{-1})	SCOD inc. (%)	SCOD/ TCOD (%)	S_D (%)
Untreated	19.8 (1.0)	0.31 (0.2)	n.d	2	n.d.
2 h (20–47 °C)	21.0 (2.2)	0.51 (0.3)	65	2	1
1.7 h (50–54 °C)	21.8 (2.2)	1.29 (0.1)	316	6	5
3.8 h (50–57 °C)	21.6 (2.2)	2.5 (0.2)	706	12	11
Freeze-thaw	20.4 (2.1)	4.1 (0.2)	1223	20	18
n.d. = not determined.					

approximately double the yield from AVRs at 20 °C (Fig. 3C, Table 4).

3.2.2. Effects of pretreatments

Digestion of both freeze-thaw and thermally pretreated MB produced methane faster than digestion of the untreated MB at 20 °C (Fig. 3D). In experiment 2, the methane yield fluctuated during the fill, probably due to varying loading, but was always higher from the pretreated MBs. During the fill, thermally pretreated MB produced 32% (136 L CH$_4$ kg VS$^{-1}_{added}$) and freeze-thaw MB yielded 50% (155 L CH$_4$ kg VS$^{-1}_{added}$) more methane than the untreated MB (103 L CH$_4$ kg VS$^{-1}_{added}$), which had a the same yield as that at 20 °C in experiment 1. After the post-MB addition period (days 79–130), the ultimate methane yields for pretreated MB were 23–27% more than for the untreated MB control, which again the same as the result in experiment 1 (Fig. 3D, Table 4).

Freeze-thaw MB was also studied in the CSTRs (days 104–130) by changing the feed from untreated MB to freeze-thaw MB but maintaining the same OLR and HRT. Addition of freeze-thaw MB increased the methane yield by 14%, averaging 205 L CH$_4$ kg VS$^{-1}_{added}$ (Fig. 3C, Table 4). It must be noted that the CSTR was operated with freeze-thaw MB for a period of only 2 HRTs, and had not necessarily reached steady state.

3.3. Digestion parameters

During experiment 1 the pH (liquid fraction) of all the AVRs declined from initial 7.7–8.2 to 7.0–7.5. The higher initial pH at the beginning of the experiment was probably due to the addition of NaHCO$_3$ buffer to prevent acidification. In experiment 2, pH levels ranged from 6.7 to 7.1 throughout the experiment. In the CSTRs, the pH was constant at approximately 7.2.

In the digestion of untreated MB in the AVRs (experiment 1), SCOD reached the highest value at the early stage of the experiment (day 30), with 2.9 g L^{-1} at 20 °C, and 1.0 g L^{-1} at 37 °C. At the end of fill 1 (day 64), SCOD had decreased to 1.6 g L^{-1} at 20 °C and 0.5 g L^{-1} at 37 °C. At the end of fill 2 (day 93), SCOD was about 1.0 g L^{-1} at both temperatures. In the CSTRs at 37 °C, SCOD remained at approximately 0.5 g L^{-1} throughout the experiment (measured on days 33, 67, 88 and 130) (Table 5).

With freeze-thaw pretreatment COD solubilisation was 18% (Table 3).

3.2. Methane production in AVRs and CSTRs

3.2.1. Effects of temperature

In experiment 1, the methane production in the AVRs was low during the first 15 and 25 d at 37 °C and 20 °C, respectively. Subsequently, methane production increased in both AVRs, but at a slower rate at 20 °C than at 37 °C. During the post-MB addition period (days 93–175), methane production at 20 °C decreased slowly, while at 37 °C, the methane production rate decreased immediately after feeding stopped (Fig. 3)A.

The methane yields at end of fill 2 were 101 L CH$_4$ kg VS$^{-1}_{added}$ and 225 L CH$_4$ kg VS$^{-1}_{added}$ at 20 °C and 37 °C, respectively (experiment 1). The ultimate methane yields after the post-MB addition period were 180 L CH$_4$ kg VS$^{-1}_{added}$ at 20 °C and 273 L CH$_4$ kg VS$^{-1}_{added}$ at 37 °C (Fig. 3A, Table 4). The ambient temperature AVRs had a comparable methane yield to the AVRs at 20 °C (experiment 2) when temperatures were above 18 °C (day 42), but the methane yield declined when the temperature decreased to 16 °C and almost ceased below 15 °C (day 95) (Fig. 3B). The methane yield of the ambient temperature AVRs averaged 83 L CH$_4$ kg VS$^{-1}_{added}$ (16–18 °C) during the fill, and the ultimate methane yield was 121 L CH$_4$ kg VS$^{-1}_{added}$ after post-MB addition period (Table 4).

In the CSTRs at 37 °C, the methane yield was 180 L CH$_4$ kg VS$^{-1}_{added}$, which was lower than that during the fill from comparable AVRs at 37 °C (225 L CH$_4$ kg VS$^{-1}_{added}$), but
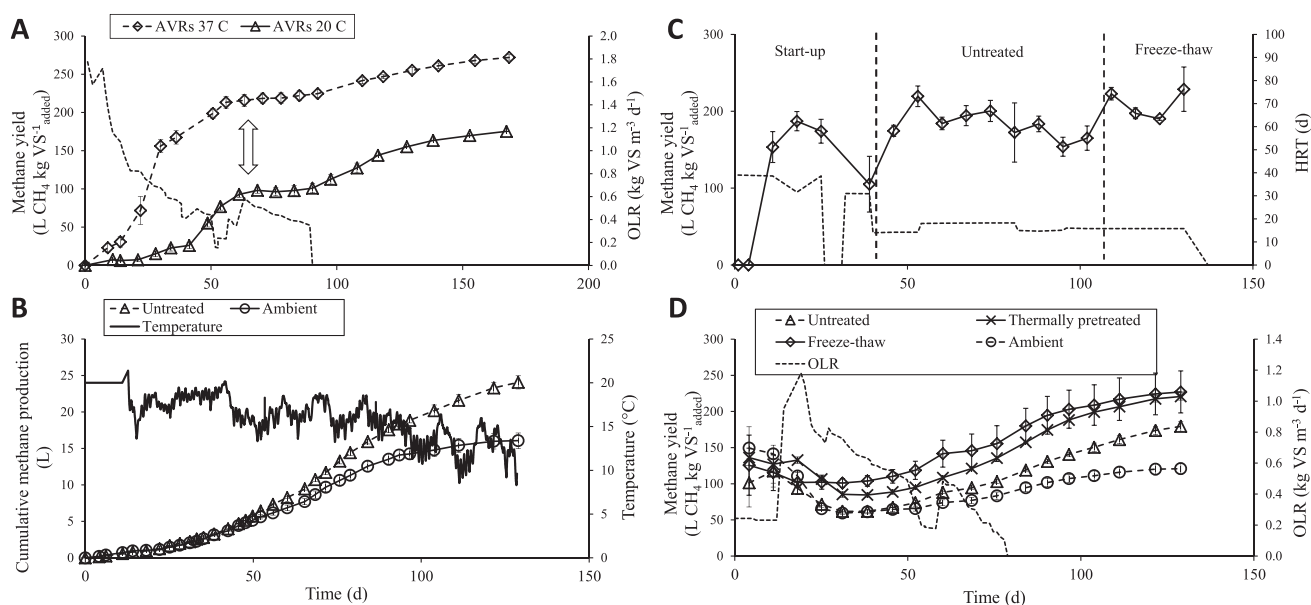
Fig. 3 — Methane productions from microalgae. (A) Total methane production per total VS fed in AVRs at 20 °C and 35 °C. Arrow = liquid withdrawal, (B) Cumulative methane production in AVRs at 20 °C and ambient temperature. Solid line shows the reactor temperature, (C) Average weekly methane yield in CSTR from MB and freeze thaw MB (OLR 1.0 g VS $d^{-1} m^{-3}$) and (D) Total methane production per total VS fed when pretreated and untreated MB were used at 20 °C and at ambient temperature. Error bars are standard deviations from three parallel reactors.

Moreover, the VFA concentrations in the AVRs and CSTRs at 37 °C were <0.1 g L^{-1} throughout the experiment. However at 20 °C AVRs in experiment 1 after fill 1 (day 64), the VFA concentration was 0.78 g L^{-1} (as COD) (which was 48% of the SCOD and made up of approximately equal shares of acetic and propionic acids). After fill 2 (day 93), the VFA concentration had decreased to 0.22 g L^{-1} (25% of the SCOD). In experiment 2, the untreated (20 °C) and ambient AVRs had VFA concentrations that were constantly <0.1 g L^{-1} (Table 5).

Both pretreated MB AVRs had SCOD concentrations that were 2-fold—3-fold higher on day 45 (1.35—1.4 g L^{-1}) and day 78 (0.74 g L^{-1}), than those AVRs with untreated MB (0.31—0.41 g L^{-1}). On day 45, VFA concentrations were

0.60 g L^{-1} (45% of SCOD) and 0.53 g L^{-1} (37% of SCOD) in freeze-thaw and thermally pretreated MB fed AVRs, respectively. Acetate made up 70—74% of VFAs and the rest was mainly propionic acid. However, at the end of the fill (day 78), the VFA concentration was <0.1 g L^{-1}, both in pretreated and untreated reactors (Table 5).

3.4. Characteristics of solid and liquid fractions in the AVRs

After fill 1 (and the post-MB addition period) in experiment 2, the solid fraction volume in all of the AVRs was 51—59% of the total liquid volume, except with freeze-thaw MB, which was

	Methane yield	Ultimate methane yield	Ultimate methane yield	VS-removal	TS-removal
	(L CH$_4$ kg VS$_{added}^{-1}$)	(L CH$_4$ kg VS$_{added}^{-1}$)	(L CH$_4$ kg VS$_{removed}^{-1}$)	(%)	(%)
Experiment 1 (AVRs)					
Untreated 20 °C	101 (95—107)	180[a]	462[a]	40 (39—40)	32 (32—32)
Untreated 37 °C	225 (6)	273 (4)	581 (13)	47 (1)	38 (1)
Experiment 2 (AVRs)					
Untreated 20 °C	103 (100—106)	179 (172—186)	504 (503—506)	36 (34—37)	32 (30—34)
Thermally pretreated 20 °C	136 (133—138)	221 (215—226)	539 (527—551)	41 (41—41)	36 (36—36)
Frozen 20 °C	155 (25)	227 (30)	540 (72)	42 (1)	37 (1)
Untreated ambient	83 (11)	121 (16)	511 (86)	24 (2)	21 (2)
CSTRs					
Untreated 37 °C	179 (17)	n.a.	560 (52)	32 (1)	23 (1)
Frozen 37 °C	205 (21)	n.a.	527 (53)	39 (1)	31 (1)

Table 4 — Methane yields and solid removals. Methane yields from untreated and pretreated MB in AVRs and CSTRs. Enclosed in parentheses are standard deviations from three parallel reactors or both results in case of two parallel reactors.

n.a. = not applicable.
[a] No parallels.

Table 5 – The characteristics of liquid and solid fractions of reactors.

	Experiment 1		Experiment 2				
	AVRs 20 °C	AVRs 37 °C	Untreated (20 °C)	Thermally pretreated (20 °C)	Freeze-thaw (20 °C)	Ambient (8–20 °C)	CSTRs (37 °C)
Liquids (fill 1)							
TS (%)	0.37 (0.01)	0.30 (0.01)	0.10 (0.01)	0.13 (0.01)	0.12 (0.01)	0.10 (0.01)	1.33 (0.01)
VS (%)	0.16 (0.01)	0.10 (0.01)	0.02 (0.01)	0.03 (0.01)	0.03 (0.01)	0.03 (0.01)	0.91 (0.01)
VS/TS (%)	43 (1)	34 (1)	24 (2)	26 (1)	27 (2)	28 (6)	68 (1)
SCOD (g L^{-1})	1.6 (0.2)	0.5 (0.1)	0.35	0.74	0.75	0.31	0.45 (0.1)
TVFA (g L^{-1})	0.7 (0.1)	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
Liquids from total mass (%)	41	45	49 (46–50)	48 (47–49)	55 (1)	46 (1)	n.a.
Liquids (fill 2)							
TS (%)	0.25 (0.02)	0.25 (0.03)	n.a.	n.a.	n.a.	n.a.	1.37 (0.03)
VS (%)	0.07 (0.01)	0.10 (0.02)	n.a.	n.a.	n.a.	n.a.	0.93 (0.02)
VS/TS (%)	26 (1)	38 (3)	n.a.	n.a.	n.a.	n.a.	68 (1)
SCOD (g L^{-1})	0.9 (0.1)	1.1 (0.2)	n.a.	enrn.a.	n.a.	n.a.	0.5 (0.1)
TVFA (g L^{-1})	0.22 (0.02)	<0.01	n.a.	n.a.	n.a.	n.a.	<0.02
Liquids from total mass (%)	28–29	33 (1)	n.a.	n.a.	n.a.	n.a.	n.a.
TKN mg L^{-1}	690 (90)	870 (110)	410 (50)[a]	710 (90)[a]	750 (90)[a]	380 (50)[a]	1090 (20)
NH$_4$ mg L^{-1}	780 (70)	950 (80)	460 (40)[a]	720 (60)[a]	650 (60)[a]	390 (40)[a]	500 (70)
Total P mg L^{-1}	170 (30)	150 (30)	130 (20)[a]	180 (30)[a]	170 (30)[a]	100 (20)[a]	180 (30)
Dissolved P mg L^{-1}	180 (20)	140 (20)	140 (20)[a]	250 (20)[a]	250 (20)[a]	140 (20)[a]	80 (30)
Solids (after post-MB addition stage)							
TS (%)	2.87 (0.05)	2.91 (0.04)	2.78 (0.10)	2.69 (0.06)	2.99 (0.06)	3.02 (0.16)	n.a.
VS (%)	1.87 (0.02)	1.87 (0.04)	1.95 (0.07)	1.84 (0.04)	2.03 (0.04)	2.19 (0.12)	n.a.
VS/TS (%)	65 (1)	64 (1)	70 (1)	68 (1)	68 (1)	73 (2)	n.a.
Protein (% TS)	35	31	40	38	35	43	n.a.
Fat (% TS)	7	7	10	9	12	10	n.a.
Carbohydrate (% TS)	17	24	19	21	21	19	n.a.
Ash (% TS)	35	36	20	32	32	28	n.a.
Solids from total mass (%)	71–72	67 (1)	51 (50–54)	52 (51–53)	45 (1)	54 (2)	n.a.
TKN mg L^{-1}	2400 (320)	2300 (290)	1900 (230)	1880 (230)	2400 (300)	2200 (270)	n.a.
Total P mg L^{-1}	230 (40)	250 (40)	230 (40)	240 (40)	210 (30)	250 (40)	n.a.

n.a. = not applicable.

[a] Nutrient concentrations are after the first fill in experiment 2.

slightly lower at 45%. After fill 2 (and the post-MB addition period) in experiment 1, the solid fraction volume increased to 67–72% of the total liquid volume. The solid fraction VS concentrations were more than 10-times higher than those in the liquid phase in all AVRs.

VS removal was higher in the 37 °C AVR (45%) than that in the 20 °C AVR (39%), and was also higher with pretreated MB (41–42%) than with untreated MB (34–37%). In the ambient reactors, VS removal from untreated MB was only 24% (Table 4).

The solid fractions that remained in the experiment 1 AVRs were analysed for protein, fat and carbohydrate content after the post-MB addition period. The protein content was 35% of TS for the 20 °C AVRs, and slightly lower (31%) for the 37 °C AVRs. The fat content was about 7% of TS at both temperatures. The carbohydrate content for the AVRs at 37 °C (24% of TS) was higher than that for the AVRs at 20 °C (17% of TS). In experiment 2, the protein content of the solids remaining in the AVRs was highest in the ambient reactor (43% of TS) and the untreated reactor (40% of TS), compared with those of the pretreated MB reactors (thermally pretreated: 38% of TS; freeze-thaw: 35% of TS) (Table 5).

Phosphorus (P) and nitrogen concentrations were determined in both the liquid and solid fractions of the AVRs after the post-MB addition period. The liquid fraction TKN and P were only made up of NH$_4$ and dissolved P respectively. The NH$_4$ concentration was higher for the 37 °C AVRs than for the 20 °C AVRs (950 mg L^{-1} and 780 mg L^{-1}, respectively) in experiment 2. However, both freeze-thaw and low-temperature pretreatments increased nitrogen solubility by 41–57% and phosphorus solubility by 77–84% in the AVRs at 20 °C (Table 5).

4. Discussion

4.1. Microalgal biomass

The average TS (1.7–1.9%) of the studied MB was in line with the TS of 1.0–2.4% that was previously reported for wastewater-grown MB harvested by gravity settling in digestion studies (Alzate et al., 2012; Passos et al., 2013). The wastewater-grown microalgae had low VS/TS of 71–74%. Earlier, VS/TS as low as 57% had been reported for algae grown in wastewater HRAPs fed (Passos et al., 2013), while VS/TS up to 95% (González-Fernández et al., 2012a) and 85–90% (Ras et al., 2011) were common for microalgae cultivated using synthetic media. The lower organic content of wastewater

grown algal biomass may be explained by the presence of inorganic compounds in wastewater, which are gravity harvested with the MB. The high nitrogen and phosphorus content of the MB indicate the potential to concentrate and recover wastewater nutrients using microalgae.

The MB had high protein content, but low fat content, both of which were probably a consequence of the unlimited nitrogen supply from the wastewater. The ultimate methane yield of MB (273 L CH_4 kg VS^{-1}_{added}) was only 48% of the calculated theoretical potential (563 L CH_4 kg VS^{-1}_{added}), but it is within the range of 227–410 L CH_4 kg VS^{-1}_{added} reported for 21 different microalgae species by Frigon et al. (2013). Methane yields reported for microalgae, even after pretreatments, are often quite low, between 100 and 200 L CH_4 kg VS^{-1}_{added} (González-Fernández et al., 2012a,b, Passos et al., 2013). Compared to conventional wastewater treatment sludge, the highest methane yield in the present study was 1.5–4 times higher than the methane yield that Bolzonella et al. (2005) reported for waste activated sludge (70–180 L CH_4 kg VS^{-1}_{added}). Also VS reductions at 20 °C or 37 °C (34–47%) were about double those of waste activated sludge (18%).

4.2. Accumulating-volume reactors

The digestion of low solid concentrations of algae from gravity settling in CSTRs leads to low loadings and/or short HRTs, which is unsuitable since algae often require retention times of at least 30 d for digestion (Ras et al., 2011). Unmixed, accumulating-volume AVRs used in the present study enabled the separation of biomass into liquid and solid factions. The liquid faction is virtually clear and contains low concentrations of organic matter (VS, COD and VFA). The liquid faction can be drawn out when the reactor is full, leaving a longer retention time for the hydrolysis of solids. Indeed, higher methane yield and VS removal were achieved with AVRs operated at 37 °C than with comparable CSTRs with the same average OLRs. The CSTRs had stable operation at an OLR of 1.0 g VS d^{-1} m^{-3} and HRTs of 14–16 d.

The amount of organic material (and nutrients) removed with the liquid phase that is drawn out of the AVR depends on the operation of the reactor, e.g., loading rate and feeding cycle. No VFAs and negligible amounts of VS were present in the liquid phase at the end of the fill in experiment 2, but with a slightly higher loading in experiment 1, some VFAs and SCOD were still present in the liquid phase of the 20 °C AVRs after fill 1. A methane potential of 270 mL CH_4 L^{-1}_{liquid} was lost with the removed liquid phase of the 20 °C AVRs, based on the dissolved VFA concentration. This was approximately 5% of the ultimate methane yield. In addition, some dissolved methane is lost with the removed liquid as with any other reactor type. In CSTRs, where effluent removal is continuous and some of the removed effluent has just been fed into the reactor, about 5–25% of ultimate methane potential can be lost, particularly with the suspended solids (Angelidaki et al., 2006). In experiment 1, the AVRs and CSTRs both had a similar volume, but the organic loading (g VS d^{-1}) was higher in the CSTRs. Since effluent is not continuously removed from AVRs (as opposed to CSTRs), to treat the same amount of MB, the AVR volume would need to be approximately 4 times that of a CSTR. However, the loading parameters were not

optimised and a smaller volume may be feasible. The longer SRT in AVRs should reduce the need for post-MB addition period sludge stabilization that is common with CSTRs. Digestion even at 35 °C is not necessarily efficient for pathogen removal (Borowski et al., 2014), so a further disinfection step may be needed for agricultural use of digestate.

In AVRs, the SRT is different to the HRT; the retention of solids in AVRs is dependent on the total reactor volume, feeding volume and the settling properties of the MB solids. The SRTs given in the present study (128 and 91 d) are the average times that solids were accommodated in AVRs, including the post-MB addition period. These times overestimate the practical SRTs in AVRs if post-MB addition period is not included. In experiment 1, the practical average SRT would have been about 55–60 d (if three fills) and in experiment 2 approximately 75–85 d.

4.3. Effects of temperature on algae degradability

The results show that AVRs fed with microalgae can be operated at 20 °C and even down to 16 °C, but below this temperature methane production decreased markedly. Zhang et al. (2012), in their study on artificial wastewater in fixed-bed bioreactors, found that 17 °C and 15 °C were the temperature thresholds, where COD removal and methane production rapidly decreased. AD at temperatures close to 20 °C is carried out by acclimatised mesophilic microorganisms, capable of living at lower temperatures with reduced activity (Kashyap et al., 2003). An adaptation period at temperatures below 20 °C or the use of true psychrophilic microorganisms (Kashyap et al., 2003) could allow digestion of microalgae at temperatures <15 °C as shown by Heubeck and Craggs (2010) with piggery waste.

The results of the present study show that AVRs require approximately double the SRT at 20 °C compared with 37 °C, or if operated with the same SRT, the methane yield at 20 °C would be approximately half that at 37 °C. These results agree with earlier findings on anaerobic digestion temperature with different substrates (Kashyap et al., 2003). Hydrolysis, especially protein hydrolysis, may have been the rate-limiting step of AD at 20 °C, as indicated by the low VFA concentrations during experiment and high protein content in the residual solids. The low VFA concentrations in the ambient temperature AVRs even when methane production had ceased due to low temperature indicate the extremely slow hydrolysis and/ or acidogenesis. In previous CSTR studies conducted at around 20 °C, the OLRs have been typically 0.15–0.6 g VS L^{-1} d^{-1} (Kashyap et al., 2003), which are clearly lower than those measured at the early stage in the present study (1.7 g VS L^{-1} d^{-1}).

4.4. Effects of pretreatments

Both freeze-thaw and low-temperature pretreatments improved MB solubilisation by increasing the amount of SCOD in the pretreated biomass. Pretreatments also enhanced methane yield and VS removal in AVRs at 20 °C. Higher methane production of freeze-thaw MB was also found in CSTRs at 37 °C. The ultimate methane yield from AD at 20 °C increased with pretreatment of the MB, although the yields

were still slightly lower than those from untreated MB at 37 °C. This result suggests that the freeze-thaw and low-temperature thermal pretreatments make degradation faster but do not necessarily enhance overall methane production. The elevated mineralisation of phosphorus and nitrogen in AVRs with pretreated MB further indicate improved degradation. Freeze-thaw pretreatment clearly improved the settling properties of MB and thus affected the solid–liquid ratio of the biomass in the AVRs. After digestion, the volume of the solid fraction was 14% less in the freeze-thaw MB fed AVR, compared with those in untreated and thermally pretreated MB fed AVRs. It is probable that freezing breaks algae cells, and intracellular liquids are then released, with the remaining cell wall structures forming larger, better settling flocs. Hu et al. (2010) showed a similar improvement in the settling properties of wastewater sludge following freeze-thaw pretreatment.

The improved solubilisation of MB after both pretreatments may have enhanced hydrolysis and therefore, the acidogenesis steps of AD. This finding is supported by the higher VFA concentrations in AVRs with pretreated MB rather than with untreated MB, which could also explain the faster methane production. Moreover, the pretreatments may have enhanced protein degradation; since the protein content of the pretreated solids was lower than that of the untreated MB. However, the low VFA concentrations suggest that hydrolysis remained the limiting step during AD at 20 °C, rather than methanogenesis.

Previous studies have also shown that low-temperature thermal pretreatment increases microalgae SCOD. The degree of solubilisation (11% after 3.8 h at 50–57 °C) found in the present study is within the range found by Alzate et al. (2012) (9–29% after 12 h at 55 °C) for 3 different microalgal species compositions. González-Fernández et al. (2012a and 2012b) reported solubilisation degrees of 6–8% after 1 h at 90 °C and ~0.5 h at 70 °C and 80 °C. For comparison, Alzate et al. (2012) also reported thermal hydrolysis at higher temperatures (110–170 °C) to yield variable but generally higher (9–63%) solubilisation.

The 13-fold increase in SCOD after freeze-thaw pretreatment in this study is higher than that reported earlier for freeze-thawed wastewater sludge. Gao (2011) found that freeze-thaw pretreatment (24 h, −18 °C) of activated sludge increased SCOD concentration from 2- to 8-fold. Montusiewicz et al. (2010) reported similar results; freeze-thaw pretreatment of mixed sewage sludge doubled the SCOD concentration. Hu et al. (2010) showed solubilisation degrees of ~3–5% and 7.5–10.5% for wastewater sludges after 24 and 72 h of freezing (at −18 °C), respectively. Compared with these results, the degree of solubilisation (18%) achieved during 24-h of freezing in this study was relatively high.

Literature results are conflicting on improved methane production following increased algal biomass solubility from using low-temperature pretreatment of MB. Passos et al. (2013) showed a linear correlation between increasing VS solubilisation and increasing methane yield. They reported a 48% increase (from 105 to 155 L CH$_4$ kg VS^{-1}$_{added}$) in methane yield for the same microalgal/bacterial biomass after a 10-h pretreatment at 75 °C. However, Alzate et al. (2012) observed even decreased (−3 to −13%) methane yields for 2 pure MB cultures, despite a 9–21% increase in COD solubilisation following 12 or 24 h thermal pretreatment at 55 °C. The authors concluded that low-temperature pretreatment at 55 °C is a biological process, requiring the presence of a specific bacterial population. Montusiewicz et al. (2010) reported that freeze-thaw pretreatment increased the methane production from wastewater sludge by 36% (based on VS removal), which is comparable to the 27% increase in methane yield found in the present study (based on VS added). Pretreatments appeared to have a more effect on MB digestability at 20 °C than at 37 °C, particularly because protein hydrolysis is slow at 20 °C and could be enhanced with pretreatments. Despite freeze-thaw's pretreatment effects, freezing volumes of low solid substrate is energy consuming and not a realistic pretreatment option, unless a method is discovered to take advantage of the winter season in cold-climate zones.

5.   Conclusions

- Ultimate methane yield (273 L CH$_4$ kg VS^{-1}$_{added}$) and VS destruction (47%) of microalgae grown in wastewater fed HRAP are up to two times higher than those of activated sludge from conventional wastewater treatment.
- Unmixed, anaerobic accumulating-volume reactors that digest microalgae can have equal or higher methane yields than those of conventional CSTRs at 37 °C. However, a larger reactor volume is required.
- Gravity sedimentation of solids from the liquid phase in the AVR leads to longer SRTs (up to 85 d) for low solid microalgae feeds. The clear liquid phase is removed when the digester is full, while the settled solids are only removed when they take up more than half of the reactor volume. Both fractions could be used as fertilizers but pathogen safety needs to be ensured.
- Methane yields at low digestion temperatures (16–20 °C) were 37–66% of the yields achieved with the traditional mesophilic digestion temperature (~37 °C).
- Low-temperature thermal (at 50–56 °C) and freeze-thaw pretreatments enhanced microalgae digestibility (32–50% higher methane yield) and mineralisation (41–84% improvement) of nitrogen and phosphorus. In particular, protein hydrolysis of the pretreated microalgae was faster in AD at 20 °C.

Competing interests

The authors declare that they have no competing interests.

Authors' contributions

Viljami Kinnunen participated in the design of the study, carried out the laboratory work, participated in the data interpretation, drafting and completion of the manuscript. Rupert Craggs conceived the study, participated in the data interpretation and did final proof read of the manuscript. Jukka Rintala participated data interpretation, drafting and

completion of the manuscript. All authors read and approved the final manuscript.

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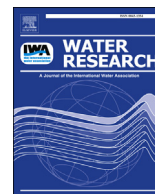
III

MESOPHILIC ANAEROBIC DIGESTION OF PULP AND PAPER INDUSTRY BIOSLUDGE–LONG-TERM REACTOR PERFORMANCE AND EFFECTS OF THERMAL PRETREATMENT

by

Mesophilic anaerobic digestion of pulp and paper industry biosludge—long-term reactor performance and effects of thermal pretreatment

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ABSTRACT

The pulp and paper industry wastewater treatment processes produce large volumes of biosludge. Limited anaerobic degradation of lignocellulose has hindered the utilization of biosludge, but the processing of biosludge using anaerobic digestion has recently regained interest. In this study, biosludge was used as a sole substrate in long-term (400 d) mesophilic laboratory reactor trials. Nine biosludge batches collected evenly over a period of one year from a pulp and paper industry wastewater treatment plant had different solid and nutrient (nitrogen, phosphorus, trace elements) characteristics. Nutrient characteristics may vary by a factor of 2—11, while biomethane potentials (BMPs) ranged from 89 to 102 NL CH_4 kg^{-1} VS between batches. The BMPs were enhanced by 39—88% with thermal pretreatments at 105—134 °C. Despite varying biosludge properties, stable operation was achieved in reactor trials with a hydraulic retention time (HRT) of 14 d. Hydrolysis was the process limiting step, ceasing gas production when the HRT was shortened to 10 days. However, digestion with an HRT of 10 days was feasible after thermal pretreatment of the biosludge (20 min at 121 °C) due to enhanced hydrolysis. The methane yield was 78 NL CH_4 kg^{-1} VS for untreated biosludge and was increased by 77% (138 NL CH_4 kg^{-1} VS) after pretreatment.

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1. Introduction

Biorefinery concepts, where wastes and/or by-products are used as a resource, have attracted interest in recent years. In many cases, the pulp and paper industry has resolved its water pollution issues by treating its wastewater with an aerobic activated sludge process (Stoica et al., 2009; Meyer and Edwards, 2014). However, wastewater treatment produces large volumes of waste-activated sludge (referred as biosludge). Stoica et al. (2009) reported a yearly biosludge production of about 2900—4000 t/mill as total solids (TS) for three Swedish pulp and paper mills. As a consequence, sludge management is one of the major costs (up to 50—60%) of the wastewater treatment process (Mahmood and Elliott, 2006; Meyer and Edwards, 2014). At present, biosludge is often dewatered and incinerated, which is not energetically favorable due to the high energy consumption of dewatering. Further, the solid content of the

dewatered biosludge remains low (18—50% TS), decreasing the heating value (Stoica et al., 2009). Incineration may not be the best utilization option if nutrient recovery from biosludge is desired, as nitrogen is lost in the incineration process and phosphorus recovery from ashes has proven challenging, mainly due to impurities (Reijinders, 2014). Recently, pulp and paper biosludge has been suggested for use in algae cultivation after anaerobic digestion (AD) in microalgae-utilizing biorefinery concepts (Kouhia et al., 2015).

In addition to methane production, the advantage of AD for sludge treatment is that the costly dewatering step is not necessarily required. AD is a conventional technology used around the world to stabilize municipal wastewater treatment plant sludges but has rarely been applied for pulp and paper industry biosludge. Pulp and paper industry biosludge has very different characteristics compared with municipal biosludge, particularly its high content of lignocellulosic material, which hinders the anaerobic degradability of the former. The lignin and cellulose contents of pulp and paper biosludge have been reported to be 36—50% and 19—27% TS, respectively, while these contents are usually <1% TS in municipal biosludge (Meyer and Edwards, 2014). The characteristics of wastewater and biosludge may vary between mills (e.g., according

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to the pulp and paper processes, raw materials, and wastewater treatment procedures used on site) (Bayr and Rintala, 2012; Ekstrand et al., 2013). However, the raw material mixture and operational parameters both in pulp and paper mill processes and wastewater treatment (e.g., nutrient dosing in wastewater treatment) are also likely to change over a longer period of time. The literature data are scarce, but it is possible that the characteristics of biosludge even from the same mill may vary over time and may potentially affect the AD if applied for sludge treatment.

Because of its low degradability, methane yields from biosludge with a high lignocellulosic content are usually low. Meyer and Edwards (2014) reviewed the biomethane potentials (BMP) reported for pulp and paper industry biosludges, and only two out of thirteen BMPs exceeded 100 L CH_4 kg^{-1} VS. Karlsson et al. (2011) studied biosludges from six Swedish pulp and paper mills, and the BMPs were 43–155 NL CH_4 kg^{-1} VS after 20 days incubation. In comparison, BMPs for municipal biosludge are often around two times higher, 200–250 L CH_4 kg^{-1} VS (Girault et al., 2012; Wang et al., 2014). The low methane yield and slow degradability requiring a long hydraulic retention time (HRT) have hindered the economical sustainability of pulp and paper industry biosludge AD (Mahmood and Elliott, 2006).

Various pretreatment methods have been screened to enhance the methane production of lignocellulosic biosludge, with thermal pretreatments being among the most promising technologies (Wood et al., 2009; Saha et al., 2011; Bayr et al., 2013). Thermal pretreatments for pulp and paper industry biosludges have been suggested to require temperatures above 150 °C, which particularly improve the hydrolysis of hemicellulose (Hendriks and Zeeman, 2009; Fernandez-Cegri et al., 2012). Lignin solubilization begins at 160 °C, but inhibitive phenolic compounds are also formed from lignin (Hendriks and Zeeman, 2009). Enhanced methane production from pulp and paper industry biosludge has been demonstrated at 150 °C (Bayr et al., 2013) and with microwave pretreatment at 75–175 °C (Saha et al., 2011). Several studies with municipal biosludge show that thermal pretreatment at temperatures <150 °C (e.g., at 121 °C) can also improve methane production (Bougrier et al., 2008; Carrére et al., 2010), but lower pretreatment temperatures are rarely studied for pulp and paper industry biosludge. A lower pretreatment temperature would reduce the input energy and prevent the formation of phenolic compounds from lignin.

Most previous AD studies with pulp and paper industry biosludges have been conducted with batch BMP assays. Only a few continuous studies have reported on the AD of pulp and paper industry biosludges without dewatering (Meyer and Edwards, 2014), although at least one full-scale reactor has been treating dewatered pulp and paper mill biosludge in Norway (Kepp et al., 2000). Compared to batch assays, continuous studies offer better understanding of the effect of HRT and the organic loading rate (OLR) on the AD process with possibly varying biosludge properties. Long-term studies could reveal the effect of varying biosludge properties on AD and the potential adaptation of microorganisms to inhibitive substances (Chen et al., 2008).

In this study, which was conducted using a mesophilic AD process and pulp and paper industry biosludge, the following three objectives were set: 1. Determine the long-term performance of mesophilic AD of biosludge in relation to varying substrate characteristics and different HRTs; 2. Find out how thermal pretreatment temperature affects methane production potential; and 3. Specify how implementation of thermally pretreated biosludge affect the performance of continuous AD. Finally, the filtered digestate produced in this study was used for algae cultivation as reported elsewhere (Polishchuk et al., 2015).

2. Materials and methods

2.1. Biosludge and inocula

Biosludge originated from a wastewater treatment plant that treats pulp and paper industry wastewater. Incoming wastewater at this treatment plant included a minor fraction (<10% of volume) of municipal wastewater. During the 400-day study period, a new biosludge batch (~70 L in a 100-L container) was obtained every second month, for a total number of nine batches. The solid concentration of feed sludge was increased from the second biosludge batch onwards to achieve higher organic loading in reactor trials. The solid concentration was increased by settling; the biosludge was kept in a 100-L container for 24 h, after which the liquid fraction (ca. 50% of the volume) was removed. The biosludge was stored at 7 °C before use.

The inoculum for the reactor trials and pretreatment screening BMP assay was mesophilically digested municipal sewage sludge from the Viinikanlahti Wastewater Treatment Plant (Tampere, Finland). All other BMP assays were conducted using digestate from the present experimental reactors (collected on days 136, 205, 289, and 400) as the inoculum.

2.2. Pretreatments

Thermal (at 80 °C, 105 °C, 121 °C, and 134 °C) pretreatments were screened to improve the degradability of biosludge. In the low thermal pretreatment condition, 500 ml of sludge in a loosely (not gas tight) closed 1-L glass bottle was warmed to 80 °C in a water bath (7 min) and subsequently kept at 80 °C in an incubator for two hours. The sludge was allowed to cool at room temperature (two hours). In the thermal pretreatment group, 500 ml of biosludge were autoclaved (KSG Sterilisatoren GmbH) in a loosely closed 1-L glass bottle at three different temperatures. The temperature was increased to 105 °C, 121 °C, and 134 °C after 36, 45, and 50 min, respectively, and kept there for 20 min before cooling to room temperature (about three hours). The pressure increased to 2.2 bars (gauge pressure) in 30 min with all treatments (back-up pressure with air and self-generated steam pressure) and was slowly released within 30 min while the sludge cooled.

2.3. BMP assays

BMP assays were conducted in 120-ml serum-bottles with a working volume of 60 ml at 35 °C as described by Kinnunen et al. (2014). A $VS_{substrate}$:$VS_{inoculum}$ ratio of 0.5 was used. The BMPs were determined for four of the nine biosludge batches (batches 3, 5, 7, and 9) using adapted inoculum from semi-continuous experimental reactors used in this study. In the pretreatment screening assays, the BMPs were determined for biosludge after thermal pretreatments using inoculum from a municipal wastewater treatment plant. In addition, a comparative BMP assay was conducted using digestate from the reactor trials as the inoculum (adapted inoculum).

2.4. Semi-continuous reactor trials

Three parallel 6-L, semi-continuous completely stirred tank reactors (CSTRs) (with a working volume of 5 L) were run for 400 days at 35 °C. A mechanical timed mixer (30 rpm) was on for 15 min and then off for 15 min during days 0–325; after that, the mixing was changed to continuous to avoid observed sludge flotation and gas tube clog up. The reactors were fed five days per week through a tube on the top of the reactor. Prior to feeding, the digestate was removed from the bottom of the reactor (7–10% less than the

feeding volume). The biogas was collected in 10-L aluminum gas bags. All three parallel reactors were fed with biosludge during days 0–313, after which two parallel reactors were fed biosludge during days 313–400 and one reactor was fed with thermally pretreated biosludge.

2.5. Analyses and calculations

The methane content of the biogas produced in the reactor trials was measured with a Shimadzu GC-2014 as described by Mönkäre et al. (2015). In the BMP assays, the methane concentration was assessed using a Perkin Elmer Clarus 500 GC-FID gas chromatograph (Mol-Sieve 5A PLOT 30 m × 0.53 mm column, oven, detector, and injector temperatures 100 °C, 250 °C, and 230 °C, respectively). Both GCs used helium as a carrier gas. The volume of biogas produced in the reactor trials was measured using water displacement. All results are given as normal temperature and pressure (NTP) as the temperature and atmospheric pressure in the lab were monitored on a daily basis. Volatile fatty acid (VFA) concentrations were measured using a Shimadzu GC-2010 FID gas chromatograph (helium carrier, ZB-WAX plus 30 m × 0.25 mm column, oven temperature 2 min 40 °C, ramp 20 °C/min to 160 °C, ramp 40 °C/min to 220 °C, 2 min 220 °C, detector and injector temperatures 250 °C). VFA concentrations were converted to be equivalent with sCOD concentrations.

Total Kjeldahl Nitrogen (TKN) was measured according to standard EN 13654-1:2001, ammonium (NH_4) according to APHA 4500-NH_3, TS and VS according to APHA 2540, Chemical oxygen demand (COD) according to APHA 5220 D, and phosphate (PO_4) according to ISO 6878:2004(E). The total phosphorus and elemental analyses were measured using inductively coupled plasma mass spectrometry. Lignin was analyzed according to Tappi-T 222 om-06, and total carbohydrates were assessed according to SCAN-CM 71:09. The soluble COD (sCOD), VFA, NH_4, and PO_4 were measured after filtration through a 0.45-μm membrane filter.

The COD solubilization degree (S_D) of the biosludge after pretreatment was calculated using Eq. (1), as described in Donoso-Bravo et al. (2011).

$$S_D = \frac{sCOD - s_0COD}{tCOD - s_0COD} * 100 \qquad (1)$$

where sCOD is the soluble COD after pretreatment, s_0COD is the soluble COD in the untreated biosludge, and tCOD is the total COD of biosludge.

BMPs are given as averages from triplicate assays. In reactor trials, methane yields from biosludge were calculated as weekly averages from three parallel reactors during days 0–64 (unsettled) and 65–312 (settled). From days 313–400, the methane yield for the biosludge was calculated as a weekly average from two parallel reactors; the methane yield for thermally pretreated biosludge was calculated from the same period as a weekly average from one reactor. The average methane yields from each experimental period were calculated excluding the first week. The OLR and the HRT in reactor trials were calculated as weekly averages, including the weekend (no feeding). During the weekdays while feeding was on, the OLR was higher and the HRT was shorter than the given weekly values.

Energy consumption in thermal pretreatments was estimated using Eq. (2)

$$Q = cm\Delta t \qquad (2)$$

where Q is the energy needed, c is the specific heat capacity ($J\ g^{-1}\ t^{-1}$), m is the mass, and Δt is the difference between the

original and the final temperature. Here, calculations were done using the specific heat capacity for water (4.186 $J\ g^{-1}\ t^{-1}$).

3. Results and discussion

3.1. Characteristics of biosludge

The characteristics of biosludge both before and after settling are shown in Table 1. The TS concentration varied from 0.7 to 1.5% in the nine biosludge batches collected over a period of one year. The TS of the biosludge was increased to 2.5–4.3% with settling conducted to allow an increase in the OLR without shortening the HRT in reactor trials. The settling properties of biosludge varied (see Supporting information), explaining the different solid contents of settled biosludge. In addition to solids, the elemental composition between biosludge batches showed notable variation. The highest phosphorus content was seven times higher than the lowest (1.2–8.6 g kg^{-1} TS, 34–200 mg L^{-1} in settled biosludge), and the highest nitrogen content doubled compared to the lowest (41–81 g kg^{-1} TS, 1.3–1.8 g L^{-1} in settled biosludge). In addition to these main nutrients, the contents of some trace elements also alternated strongly in the three batches that were analyzed for elements: Al (7–23 g kg^{-1} TS), Ca (4.6–14 g kg^{-1} TS), and Mg (0.25–2.8 g kg^{-1} TS) (see Supporting information).

Biosludge nitrogen content in the present study was comparable with 33–77 g kg^{-1} TS, which was reported for 15 pulp and paper mill biosludge samples (Elliott and Mahmood, 2007). It must be noted that even though the TKN concentration of the settled biosludge was 1.3–1.9 g L^{-1}, less than 10% was in NH_4 form (<90 mg L^{-1}). The total phosphorus concentration varied considerably between the three measured biosludge batches, but the concentrations were lower than 5–28 g kg^{-1} TS, the value that was reported earlier for pulp and paper industry biosludge (Elliott and Mahmood, 2007). Nitrogen and phosphorus are often added to facilitate the biology of the activated sludge process, as was also the case in the wastewater treatment process from which the studied

Table 1
Characteristics of pulp and paper mill biosludge and settled biosludge. Values are minimums and maximums measured from nine different sludge batches, except for those given in footnotes. Standard deviations are enclosed in parentheses when applicable (three replicates).

	Biosludge	Settled biosludge
TS (%)	1.1–1.5	2.5–4.3
VS (%)	0.7–1.0	1.8–3.2
VS/TS (%)	65–71	68–78
Lignin (% TS)	44 (5)[a]	44 (5)[a]
Carbohydrates (% TS)	30 (2)[a]	30 (2)[a]
TKN (g kg^{-1} TS)	81 (3)[b]	44–81
TKN (mg L^{-1})	560 (20)[b]	1280–1830
NH_4 (mg L^{-1})	74 (5)[b]	27–81
Total P (g kg^{-1} TS)	n.m.	1.2–8.6[c]
Total P (mg L^{-1})	n.m.	34–200[c]
PO_4–P (mg L^{-1})	6 (1)[b]	6–20[d]
pH	7.4[b]	7.2–7.4
TCOD (g L^{-1})	12 (1)[b]	29–35[e]
SCOD (g L^{-1})	0.9 (0.1)[b]	0.6–1.4[e]
$BMP_{35\ d}$ (NL $CH_4\ kg^{-1}$ VS)	93 (3.7)[f]	89–102[g]
$BMP_{35\ d}$ (NL $CH_4\ kg^{-1}$ TS)	54 (2.6)[f]	63–75[g]
$BMP_{35\ d}$ (NL $CH_4\ kg^{-1}$ ww)	0.6 (0.1)[f]	1.6–2.5[g]

n.m. not analyzed.
[a] Biosludge batch 5 only.
[b] Biosludge batch 1 only.
[c] Biosludge batches 4, 5 and 6.
[d] Biosludge batches 1, 2, 3 and 9.
[e] Biosludge batches 1, 7 and 9.
[f] Biosludge batch 7 only.
[g] Biosludge batches 3, 5, 7 and 9.

biosludge originated. The varying dosing of nitrogen and phosphorus to the activated sludge process may be one factor that explains the concentration changes between biosludge batches. Trace elements, such as Ca, Mg, Al, and Fe, are important micronutrients for anaerobic microorganisms, but at high concentrations they may also cause inhibition. Although strongly varying, the trace element concentrations in the present study were below the reported inhibitory levels (Chen et al., 2008).

Despite the changing characteristics of different biosludge batches, the BMPs remained quite similar, ranging from 85 to 102 NL CH$_4$ kg VS^{-1} (Fig. 1A) without any difference between settled and non-settled biosludge. The low BMPs of biosludge from different pulp and paper production processes (kraft, BCTMP, BCTMP/TMP, mechanical, and chemical) have been widely reported earlier (Meyer and Edwards, 2014). Bayr et al. (2013) found BMPs of 50–100 NL CH$_4$ kg^{-1} VS for biosludge, while Karlsson et al. (2011) reported higher potentials (100–200 NL CH$_4$ kg^{-1} VS) for six different pulp and paper industry biosludges after long incubation times (89–114 days). Pulp and paper industry biosludge BMPs remain low apparently due to the high concentrations of lignin (about 44% of TS in the present study).

3.2. The effect of thermal pretreatments on biosludge characteristics

The effect of thermal pretreatments on biosludge solubility and methane production was first screened using thermal (two hours at 80 °C, 20 min at 105 °C, 121 °C, or 134 °C) pretreatments. The COD solubilizations (described using S$_D$) and BMPs for untreated and pretreated biosludge are presented in Table 2.

The solubilization increased for all four tested treatment temperatures (Table 2) and also with increasing treatment

temperatures. The highest S$_D$ of 22% was achieved after treatment at 134 °C, while the S$_D$ was lowest (6%) after treatment at 80 °C. The BMPs increased correspondingly with increased S$_D$; the BMP after pretreatment at 134 °C was 88% higher (124 NL CH$_4$ kg^{-1} VS) than that for sludge without pretreatment (66 NL CH$_4$ kg^{-1} VS). The difference in methane production between untreated and pretreated biosludge occurred within the first 10 days of BMP assays with all treatments, when most of the methane was formed (Fig. 1B). Indeed, 140% and 100% more methane (108 and 90 NL CH$_4$ kg^{-1} VS) was formed during the first 10 days from biosludge treated at 134 °C and 121 °C, respectively, compared with untreated biosludge (45 NL CH$_4$ kg^{-1} VS). When adapted inoculum from experimental reactors was used as the inoculum in BMP assays, the BMPs were about 15–24% higher compared with assays that used non-adapted inoculum (Fig. 1B). The methane production was faster with the adapted inoculum, as the difference in methane production occurred again within the first 10 days of digestion.

Interestingly, the thermal treatment at 134 °C increased the pH of the biosludge from about 7.5 to 8.4 when the pH did not change during other treatments. The treatment at 134 °C also had other effects, which did not occur at other temperatures, such as stronger foaming and failure of PO$_4$ analysis (formation of a precipitate). An increased pH and also foaming could follow protein desorption, as suggested by Bougrier et al. (2008) after a similar observation (increased pH) following thermal pretreatment of municipal biowaste. This theory is supported by the slight decrease of TKN content (from 51 to 49 g kg^{-1} TS) in biosludge treated at 134 °C. Further, the NH$_4$ concentration of biosludge increased in all other pretreatments from an initial 70 mg L^{-1} to 138–160 mg L^{-1}, but it only went up to 111 mg L^{-1} after treatment at 134 °C. It is likely that the increased pH together with the high temperature caused increased ammonia evaporation. The color of the filtrate from pretreated sludges darkened gradually with increasing temperature, becoming almost black after treatment at 134 °C (see Supporting information).

The present study shows that enhancement in BMPs were achieved with pretreatments at 105–134 °C. The increased solubilization and higher BMPs are in accordance with earlier findings, with a 55–280% (Wood et al., 2009) and 68% (Saha et al., 2011) increase in BMPs after thermal treatment at higher temperatures (170 °C and 175 °C) of pulp and paper mill sludges. Unlike this study, however, Saha et al. (2011) reported that microwave pretreatment improves the BMP already at 75 °C. The same authors also found that treatment at 125 °C was nearly as efficient as treatment at 175 °C. However, neither this study nor most previous studies have found the BMPs of pulp and paper industry biosludge to exceed 200 NL CH$_4$ kg^{-1} VS (Meyer and Edwards, 2014). To our knowledge, only Saha et al. (2011) reported a BMP as high as 290 NL CH$_4$ kg^{-1} VS for pretreated (microwave at 175 °C) pulp and paper biosludge, which likely indicates a difference between sludges, as untreated sludge also had a higher BMP in their study. The lignin and cellulose concentrations were not measured after pretreatments, so it is unclear whether the increased solubility was due to the breakdown of these compounds or the breakdown of a microbial biomass. As microbial biomass is a major component of biosludge, it is likely that also increased solubility at relatively low temperature pretreatments was originated from microbes. However, Saha et al. (2011) observed that soluble sugars from pulp and paper biosludge increased significantly when the pretreatment temperature exceeded 100 °C, suggesting solubilization of cellulose to some extent.

The higher BMPs obtained when using an inoculum from reactor trials was used to show that anaerobic microorganisms adapted to the pulp and paper industry. The adaptation ability of anaerobic microorganisms for various environments has been
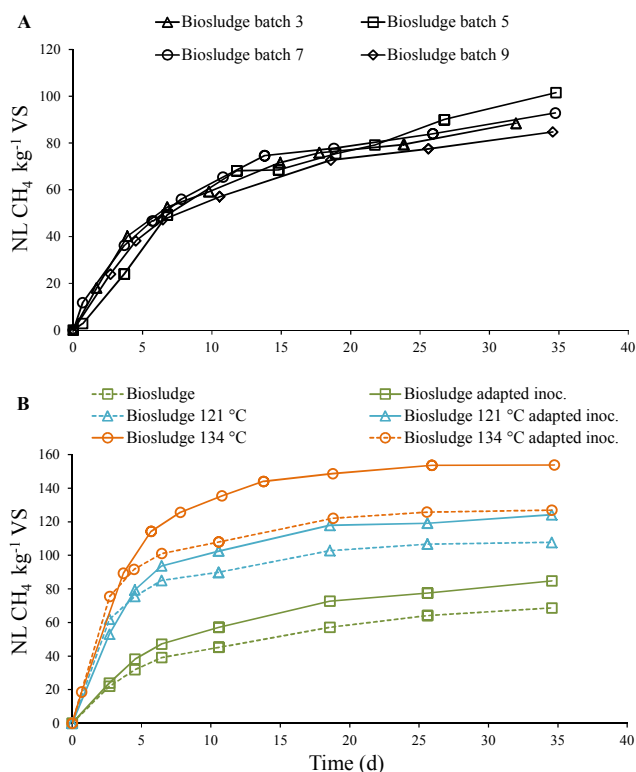

Fig. 1. Cumulative methane production during BMP assays. Methane production; (A) from four pulp and paper industry biosludge batches, collected different times; (B) from untreated and pretreated biosludge using both adapted and non-adapted inocula.

Table 2
The effect of thermal pretreatments on COD solubilization degree (S_D), pH, nutrient mineralization, and BMPs of pulp and paper biosludge. Standard deviations are shown in parentheses when applicable (three replicates).

	Untreated	2 h 80 °C	20 min 105 °C	20 min 121 °C	20 min 134 °C
TS (%)	3.1 (0.1)	3.1 (0.1)	3.0 (0.1)	3 (0.1)	3.3 (0.1)
VS (%)	2.3 (0.1)	2.2 (0.1)	2.1 (0.1)	2.1 (0.1)	2.4 (0.1)
VS/TS (%)	72 (1)	72 (1)	72 (1)	72 (1)	72 (0.1)
TKN (g kg^{-1} TS)	51	51	52	51	49
NH$_4$ (mg L^{-1})	67	107	99	89	19
PO$_4$ (mg L^{-1})	17	20	18	17	n.a.
pH	6.9	6.8	6.9	6.9	8.4
COD solubilisation (S_D, %)	n.a.	6	11	14	22
BMP$_{10 d}$ (NL CH$_4$ kg^{-1} VS)	45 (2)	48 (2)	77 (1)	90 (7)	108 (6)
BMP$_{10 d}$ (% change)	n.a.	7	71	100	140
BMP$_{35 d}$ (NL CH$_4$ kg^{-1} VS)	66 (1)	60 (7)	92 (4)	107 (8)	124 (4)
BMP$_{35 d}$ (% change)	n.a.	−9	39	62	88
VS removal (%)	11	9	17	23	26

n.a.: not applicable.

reported in literature (Chen et al., 2008). However, when untreated biosludge from batches 3, 5, 7, and 9 was assayed using an adapted digestate collected on days 136, 205, 289, and 400, respectively, no increasing trend in the BMPs was noticed (Fig. 1A). This result suggests that the adaptation occurred relatively quickly and a longer inoculum adaptation time did not further enhance the degradation.

3.3. Untreated biosludge in reactor trials

Operation parameters, methane yields, and digestate characteristics during 400-d reactor trials are shown in Fig. 2 and Table 3. With untreated biosludge, the OLR varied between 0.5 and 1.9 kg VS m^{-3} d^{-1}, while HRTs of 20 d (days 0–301), 14 d (days 301–365), and 10 d (days 365–400) were applied.

At the beginning of the trial, during days 1–64, the reactors were fed with unsettled biosludge that had a low solid content (TS 1.2%). During this period, both the methane yield (46–98 NL CH$_4$ kg^{-1} VS, averaging 71 NL CH$_4$ kg^{-1} VS) and especially the methane concentration of the biogas fluctuated widely (50–62%, Fig. 2B). After the introduction of settled biosludge (TS 2.0–3.4%), the methane yield averaged 74–79 NL CH$_4$ kg^{-1} VS; the methane concentration was 62–63% during days 65–364. On day 365, when

Fig. 2. Anaerobic digestion of pulp and paper industry biosludge and thermally pretreated biosludge. Weekly average methane yields, OLR and HRT (A), biogas methane concentrations (B) during semi-continuous reactor trials. The arrow indicates the time when settled biosludge feed was started (non-settled biosludge until that day), dashed lines indicate the time when HRTs were changed.

the HRT was decreased to 10 days, methane production ceased immediately within the first five days of feeding in both parallel reactors (Fig. 2A). Methane production did not recover within two weeks after the feeding was stopped on day 373. When the HRT was shortened from 20 to 14 days, the flotation of solids inside the reactor occasionally occurred, which was probably due to the higher gas production.

With seven different settled biosludge batches (days 64–364), the weekly methane yields in reactor trials differed a maximum of 19% from the averages (74 and 77 NL CH$_4$ kg^{-1} VS) of each HRT. This result strongly suggests that even the biosludge collected at varying times over a whole year had differences, especially in TS and nutrient concentrations; however, the degradability and methane yield remained the same. The average methane yield in reactor studies was about 20% lower compared with the BMP assay. This finding is a consequence of the shorter retention time and the type of process in CSTR, where a fraction of freshly introduced material has to be removed before degradation. Shortening the HRT from 20 d to 14 d did not affect the methane yield. Shortening the HRT neither increased the sCOD (<0.6 g L^{-1}) nor the VFA (<0.1 g L^{-1} as sCOD) concentrations, which both remained at a low level (Table 3). The methane yields in the present reactors were quite low, but 71–79 NL CH$_4$ kg^{-1} VS for untreated biosludge is in accordance with earlier results where methane yields from untreated biosludge from the pulp and paper industry have rarely exceeded 100 NL CH$_4$ kg^{-1} VS in reactor studies (Meyer and Edwards, 2014).

Changes in biosludge characteristics were not seen in methane production, but they did affect the digestate properties. The NH$_4$-concentration in the digestate varied between 170 and 590 mg L^{-1}, with the change being related to varying TKN concentrations (1.2–1.8 g L^{-1}) of the fed biosludge. On average, 26% of the TKN was mineralized to the NH$_4$ form. The variations of NH$_4$ and soluble P concentrations in the digestate likely originated from the activated sludge wastewater treatment process, where nitrogen and phosphorus are introduced to supply aerobic microorganisms, and the dosing causes variation in the biosludge nutrient concentrations. However, changes may also arise from process changes, varying raw materials (e.g., wood species), and seasonal variations in raw materials between the cold winter months and summer. These factors could be the subject for further study.

3.4. Thermally pretreated biosludge in reactor trials

Thermal pretreatment at 121 °C was chosen for studies in semi-continuous reactors because of the relatively high enhancement in BMP, no nitrogen losses, and brighter color of the filtrate compared to pretreatment at 134 °C (the digestate was used for algae
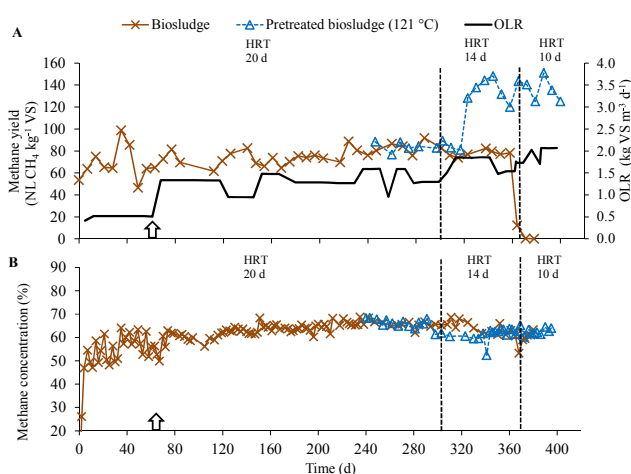
Table 3
Operation parameters, methane yields, methane concentrations, and digestate characteristics during 400 d reactor trials. When applicable, standard deviations are enclosed in parentheses.

	Biosludge				Pretreated biosludge (at 121 °C)	
Operation parameters						
Time (d)	1–64	65–301	302–364	365–400	313–364	365–400
OLR (kg VS m^{-3} d^{-1})	0.5	1.0–1.6	1.6–1.9	1.8–2.2	1.6–1.9	1.8–2.2
HRT (d)	20	20	14	10	14	10
Gas production						
Methane yield (NL CH_4 kg^{-1} VS)	70 (16)[a]	76 (8)[d]	78 (3)[f]	0	138 (11)[h]	134 (13)[i]
Methane yield (Nm^3 CH_4 t^{-1} ww)	0.6 (0.2)[a]	2.0 (0.5)[d]	2.1 (0.2)[f]	0	3.0 (0.5)[h]	2.5 (0.3)[i]
Methane conc. (%)	56 (6)[a]	63 (3)[d]	62 (2)[f]	59 (4)[f]	63 (2)[h]	63 (2)[i]
TS-removal (%)	3[a]	6–6	6–7	n.m	17	13
VS-removal (%)	9[a]	9–10	9–10	n.m	25	19
Digestate characteristics						
VFA (mg L^{-1} as sCOD)	<0.1	<0.1	<0.1	<0.1	<0.1	<0.1
SCOD (g L^{-1})	0.7 (0.1)[b]	0.6 (0.1)[e]	0.6 (0.1)[g]	0.4 (0.1)[c]	1.4 (0.1)[c]	1.6 (0.1)[c]
TKN (g L^{-1})	n.m.	1.3–1.8	1.7 (0.1)[c]	1.4 (0.1)[c]	1.5 (0.1)[c]	1.6 (0.1)[c]
TKN (g kg^{-1} TS)	170 (20)[c]	44–65	54	n.m.	58 (6)[c]	59 (6)[c]
NH_4–N (mg L^{-1})	170 (20)[c]	210–590	300–370	n.m.	496 (25)[c]	486 (24)[c]
Total P (mg L^{-1})	n.m.	126–235	n.m.	n.m.	n.m.	n.m.
Total P (g kg^{-1} TS)	n.m.	6.7–8.0	n.m.	n.m.	n.m.	n.m.
PO_4–P (mg L^{-1})	n.m.	n.m.	12 (2)[c]	18 (2)[c]	28 (3)[c]	33 (4)[c]
pH	7.0–7.2	6.9–7.2	7.2	7.0–7.1	7.0–7.2	7.1–7.2
Lignin (% TS)	n.m.	46	n.m.	n.m.	n.m.	n.m.
Carbohydrates (% TS)	n.m.	7	n.m.	n.m.	n.m.	n.m.

n.m.: not measured. Number of replicates: [a]32; [b]16; [c]3; [d]75; [e]40; [f]14; [g]8; [h]6; [i]4.

cultivation). One reactor was fed with thermally pretreated bio-sludge from day 313 onwards, while two parallel reactors were kept as a control and fed with untreated biosludge. The HRTs of all reactors were shortened from 14 d (days 313–364) to 10 d (days 365–400).

The methane yield increased immediately after the introduction of pretreated biosludge on day 313 (Fig. 2A). With an HRT of 14 days, the methane yield (138 NL CH_4 kg^{-1} VS) from thermally pretreated biosludge was 75% higher than from untreated biosludge (79 NL CH_4 kg^{-1} VS). After day 365, when the HRT was shortened to 10 days (OLR 2.2 kg VS m^{-3} d^{-1}), the methane yield from pretreated biosludge averaged 134 NL CH_4 kg^{-1} VS, nearly the same as the yield as the previous HRT of 14 d. It must be kept in mind that the methane production from untreated biosludge ceased immediately after similar shortening of the HRT (Fig. 2A). After the introduction of pretreated sludge, TS removal increased from 6% to 17% and VS removal rose from 10% to 25% (Table 3).

After introducing pretreated biosludge feed, the sCOD concentration of the digestate increased from 0.5 g L^{-1} to 1.4 g L^{-1}. When the HRT was decreased from 14 to 10 d, the sCOD in the digestate further increased to 1.6 g L^{-1} while the VFA concentration was <0.1 g L^{-1} as sCOD. The increase of sCOD with pretreated biosludge is in contrast to that of untreated biosludge, where sCOD concentrations decreased notably (from 0.6 to 0.3 g L^{-1}) and the VFA concentration remained <0.1 g L^{-1} as sCOD after the HRT was shortened to 10 days.

According to the present results, it is likely that hydrolysis was the limiting step of the AD process with the studied pulp and paper industry biosludge, while the critical HRT for the hydrolysis of untreated biosludge in CSTR was between 10 and 14 days. This result is supported by the fact that the methane concentration of the gas produced after the methane yield ceased remained high (Fig. 2B), indicating that the methanogenesis step was still occurring. As suggested by the increased COD solubility, the thermal pretreatment enhanced the hydrolysis, providing a greater amount of substrate for downstream microbial metabolism and enabling the process to take place with a lower HRT compared with untreated biosludge. However, some of the solubilized COD in the pretreatment was anaerobically non-degradable, as the sCOD

concentration in digestate increased after feeding with pretreated biosludge was started. This is supported by the clearly darker color of filtered digestate (see Supporting information), probably caused by higher concentration of soluble lignin. Darker color could hinder the use of digestate e.g. in algae cultivation. The 10-d HRT achieved after thermal pretreatment is to our best knowledge among the shortest reported HRTs for pulp and paper industry biosludge. Earlier, Puhakka et al. (1992) used short HRTs of 8 days for pulp mill biosludge. The short retention in the present study must be underlined, as the experiments were semi-continuous (the reactors were not fed during weekends), meaning that when calculated for weekdays, the HRT was as short as 7 days.

Thermal pretreatment improved the methane yield up to 77% in reactor trials, but the methane yield (138 NL CH_4 kg^{-1} VS) remained at a low level even after pretreatment. Because of the low solid concentration, particularly in the case of non-settled biosludge (TS 1.1–1.5% and 2.5–4.3% for settled biosludge), the methane yields were especially low when calculated per ton of wet sludge (0.6 Nm^3 CH_4 t^{-1} ww for non-settled and 2.0–2.1 Nm^3 CH_4 t^{-1} ww for settled biosludge). With thermal pretreatment, the methane yield of the settled biosludge was improved to 2.5–3.0 Nm^3 CH_4 t^{-1} ww, which corresponds to a maximum 10 kWh t^{-1} energy gain. However, heating the sludge from 35 °C to 121 °C consumes (calculated for water) approximately 100 kWh t^{-1}, creating a strongly negative energy balance if the majority of the heat (80–90%) is not recovered (e.g., via heat exchangers). Even with notable enhancement in the methane yield, the pretreatment could likely be energetically sustainable only if waste heat can be used or other improvements achieved with pretreatment. Pretreatment could allow energy savings due to a smaller reactor size (heating and building the reactor) because of the shorter HRT required. After improved efficiency in AD with pretreatment, the digestate quality could be improved, which includes having fewer solids, easing transportation, further processing and having potentially better quality for fertilizer use. There is also increasing interest to find a use for lignin (Thakur et al., 2014), which does not degrade in AD; however, these are topics for further research.

4. Conclusions

- The long-term (365 d) mesophilic anaerobic digestion of bio-sludge showed stable operation (having methane yield of 70–78 NL CH_4 kg^{-1} VS) with HRTs of 20 and 14 days. Stable operation was achieved despite the characteristics of nine biosludge batches varied notably in solid concentration, settling properties and, especially for nutrient concentrations. However, the low solid concentration (1.5% TS) of the biosludge caused an unstable AD process, unlike the process with higher solid biosludge (2.5–4.3% TS).
- Thermal pretreatments carried out by autoclaving the pulp and paper biosludge at 105–134 °C increased biosludge solubility and enhanced the BMPs by 39–88%, while pretreatment at 80 °C did not affect the final BMP.
- In continuous AD, thermal pretreatment step at 121 °C increased biosludge methane yield by 77% (138 NL CH_4 kg^{-1} VS) and also allowed AD with shorter HRT of 10 d (OLR 2.2 kg VS m^{-3} d^{-1}), while digestion of the untreated biosludge failed with 10-d HRT due slow hydrolysis. Shorter HRT enables smaller reactor size, decreasing energy consumption. However, overall energy balance and feasibility of thermal pretreatment requires case-specific evaluation.

Acknowledgments

Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.watres.2015.08.053.

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IV

THE EFFECT OF LOW-TEMPERATURE PRETREATMENT ON THE SOLUBILIZATION AND BIOMETHANE POTENTIAL OF MICROALGAE BIOMASS GROWN IN SYNTHETIC AND WASTEWATER MEDIA.

by

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The effect of low-temperature pretreatment on the solubilization and biomethane potential of microalgae biomass grown in synthetic and wastewater media

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HIGHLIGHTS

- *C. vulgaris* and native algae were grown in sterile and non-sterile media.
- Methane production from native microalgal biomass was 154–252 L CH_4 kg^{-1} VS.
- Low-temperature pretreatment at 80 °C increased the biomethane potential by 11–24%.
- The differences in the BMP and solubilization originate from cultivation media.

GRAPHICAL ABSTRACT
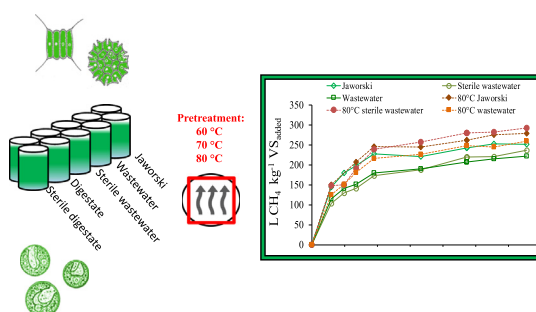
ABSTRACT

Microalgae have been suggested as a sustainable raw material for biofuel production in the form of methane via anaerobic digestion. Here, pretreatments at 60–80 °C were investigated, aiming to study the impact of algae culture media on biomethane potential and pretreatment efficiency. *Chlorella vulgaris* and mixed culture of native algae species (dominating by *Scenedesmus* sp.) were grown in synthetic medium, wastewater (sterilized and non-sterilized) and digestate from anaerobic digestion of pulp and paper biosludge (sterilized and non-sterilized). The biomethane potential for native microalgal biomass varied between 154 and 252 L CH_4 kg^{-1} VS depending on culture media. The efficiency of the low-temperature pretreatment (80 °C, 3 h) for solubilization (9–12%) of *C. vulgaris* and native algae biomass was similar for algae grown in sterilized and non-sterilized wastewater media. The pretreatment increased the biomethane potential of native algae biomass by 11–24%.

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1. Introduction

Microalgae have been widely studied as a substrate for methane production using anaerobic digestion (AD) (Chaudry et al., 2015). However, algal biomass seems to degrade poorly and produce little methane compared to theoretical values, hindering the feasibility of AD (Passos et al., 2014). The low degradability of algae is often

due to the robust cell wall, which has several layers of cellulose, hemicellulose and recalcitrant compounds (González-Fernández et al., 2012a; Passos et al., 2015); for instance *Chlorella vulgaris* and *Scenedesmus* sp. are known to have a cellulosic cell wall that is difficult to degrade (Lü et al., 2013; González-Fernández et al., 2012a, 2012b). To improve algae degradability, various pretreatment methods have been investigated aiming to solubilize microalgae biomass and therefore to enhance degradability. Increased solubilization may result from extracellular compounds, such as exopolymers and/or from the release of intracellular

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macromolecules to the soluble phase after cell disruption (Ometto et al., 2014; Passos et al., 2014).

To make methane production from microalgae economically and environmentally feasible, recent research emphasized the use of waste nutrients (e.g., wastewater) instead of conventional fertilizers to cultivate algae (Chen et al., 2015). Microalgae can be grown in wastewater (Craggs et al., 2012; Quiroz Arita et al., 2015; Chen et al., 2015) and in the liquid fraction of the digestate from AD (Hidaka et al., 2014; Morales-Amaral et al., 2015; Polishchuk et al., 2015). However, maintaining a population of a single species of alga in wastewater or digestate cultivation may be difficult. Chen et al. (2015) recommended mixed native species of algae when wastewater is used as a growth medium.

Previous AD studies of microalgal biomass were often conducted with algae grown in synthetic media or sterilized wastewater (Wu et al., 2014). Sterilized growth media may does not introduce bacterial contamination to the cultivation, whereas non-sterilized wastewater or digestate as a nutrient source adds diverse bacterial fauna to the cultivation, and subsequently to the collected algal biomass (Craggs et al., 2012; Ramanan et al., 2016; Wieczorek et al., 2015). Consequently, the impact of growth media, and bacteria in it, on anaerobic degradability of microalgae biomass is still poorly understood. For instance, the degradability and methane production from *C. vulgaris* have been shown to increase after bioaugmentation with a bacterial population (Lü et al., 2013).

Thermal pretreatments have shown promise for improving algae degradability. These treatments are often classified as low-temperature (50–100 °C) and hydrothermal (>100 °C) pretreatments. Furthermore, the temperature range of 50–70 °C is often considered as biological pretreatment (Passos et al., 2014). Biological pretreatments have been proposed to promote the activity of thermophilic and hyperthermophilic bacteria (Passos et al., 2014), and suggested to work better for an algal biomass with bacteria than with a pure algal culture (Alzate et al., 2012). The latter is likely because bacteria excrete enzymes that degrade the algae cell wall, enhancing cell lysis (Ramanan et al., 2016). Bacteria-induced algal cell lysis has been reported to aid lipid extraction (Lenneman et al., 2014). However, the positive impact of pretreatments at around 50–60 °C on methane production has often found to be modest, increasing the methane yield by only up to 12% (Passos et al., 2014) or even decreasing methane production (Alzate et al., 2012). Low-temperature pretreatments at 80–100 °C have shown more promise, by increasing methane production up to 60–220% compared with that of untreated algae (Passos et al., 2014). Passos and Ferrer (2014) calculated the positive energy balance for low-temperature pretreatments of microalgae at 75–90 °C and recently Passos et al. (2016) reported pretreatment at 80 °C for 2 h to provide maximum methane yield when 80, 115 or 150 °C with 2, 5 and 8 h of exposure time were investigated.

The aim of this study was to investigate the effect of low-temperature pretreatment (60–80 °C) on solubilization (measured using soluble chemical oxygen demand, sCOD) and on biomethane potential (BMP) of microalgae, comparing algae cultivated in synthetic medium, sterilized and non-sterilized wastewater and digestate from AD of pulp and paper biosludge. The microalgae were collected from a boreal freshwater lake, but because presence of bacteria possibly affecting the pretreatment efficiency could not be avoided with native microalgae, the pretreatment effect on the solubilization of a pure culture of *C. vulgaris,* grown in synthetic medium, wastewater and digestate, was investigated as a control. The pretreatment temperatures were chosen to cover the temperature area from suggested biological range (50–70 °C) to actual low-temperature pretreatment range (80 °C).

2. Materials and methods

2.1. Experimental setup

The study consisted of three experimental setups in which the impact of the biological and low-temperature pretreatment on the microalgae solubility and the BMP was investigated. The experimental setups are summarized in Fig. 1. In experiment 1, the impact of pretreatments at 60, 70 and 80 °C (treatment times 0–9 h), on chemical oxygen demand (COD) solubilization was investigated using a pure culture of *C. vulgaris*, cultivated in synthetic Jaworski's medium. In experiment 2, the impact of pretreatment at 60 and 80 °C (3 h), on COD solubilization of *Chlorella* grown in Jaworski media and different waste media (Jaworski's medium, wastewater, sterilized wastewater, digestate and sterilized digestate) was investigated. Finally, in experiment 3, the effect of pretreatment at 80 °C (3 h) on COD solubilization and the BMP of native microalgal biomass was investigated after cultivation in all the growth media.

2.2. Growth media

Five different media were used in this study to cultivate *Chlorella* and native microalgae. The nutrient characteristics of the growth media are shown in Table 1. Jaworski's medium was prepared according to the Culture Collection of Algae and Protozoa (CCAP). Municipal wastewater was obtained from the wastewater treatment plant at Viinikanlahti, Tampere, Finland. The wastewater was collected after an initial sieving step before any additional treatment was performed or chemical was added. Fresh wastewater was obtained for each experiment. The wastewater was filtered through a 0.45 μm filter (experiment 2) or through a 0.1 mm sieve (experiment 3), the latter to increase comparability with potential full-scale applications. The digestate was from laboratory anaerobic digester fed with biosludge from the pulp and paper industry described more detail in Kinnunen et al. (2015). The digestate was first centrifuged for 10 min at 5000 rpm, and then supernatant was filtered through a 0.45 μm filter. The growth medium was 25% of digestate in water based on preliminary dilution experiments. For the digestate and wastewater, sterilized and non-sterilized growth media were used. Sterilization was performed by autoclaving at 121 °C. Jaworski's medium was sterilized similarly in all experiments.

2.3. Microalgal biomass and inoculum

In this study, pure algae culture *Chlorella* and a mixed culture of native, boreal freshwater microalgae were used. *Chlorella* was obtained from previous studies (Lakaniemi et al., 2012) and stored at −85 °C. The native algal population was collected by taking a water sample from Lake Pyhäjärvi (June, Tampere, Finland). The algae were pre-cultivated in 250 mL Erlenmeyer flasks in 150 mL synthetic Jaworski's medium (*Chlorella*) or in sterilized wastewater (native algae). The flasks were continuously stirred at 150 rpm.

Chlorella was cultivated in four parallel 1 L Erlenmeyer flasks with 0.5 L liquid volume of Jaworski's medium (experiment 1), wastewater, sterilized wastewater, digestate and sterilized digestate (experiment 2). The flasks were continuously stirred (150 rpm) and illuminated (Osram L 18 W/965 biolux) with light intensity of 90 μmol photons m^{-2} s^{-1} (experiments 1 and 2). In experiment 3, native microalgae were grown in duplicate or triplicate 1 L glass bottles with 0.7 L liquid volume and mixed with 0.7 L min^{-1} L^{-1} airflow through an air diffuser at the bottom of the bottle. Illumination was provided with the same fluorescent lamps as in experiments 1 and 2 but with a light intensity of about
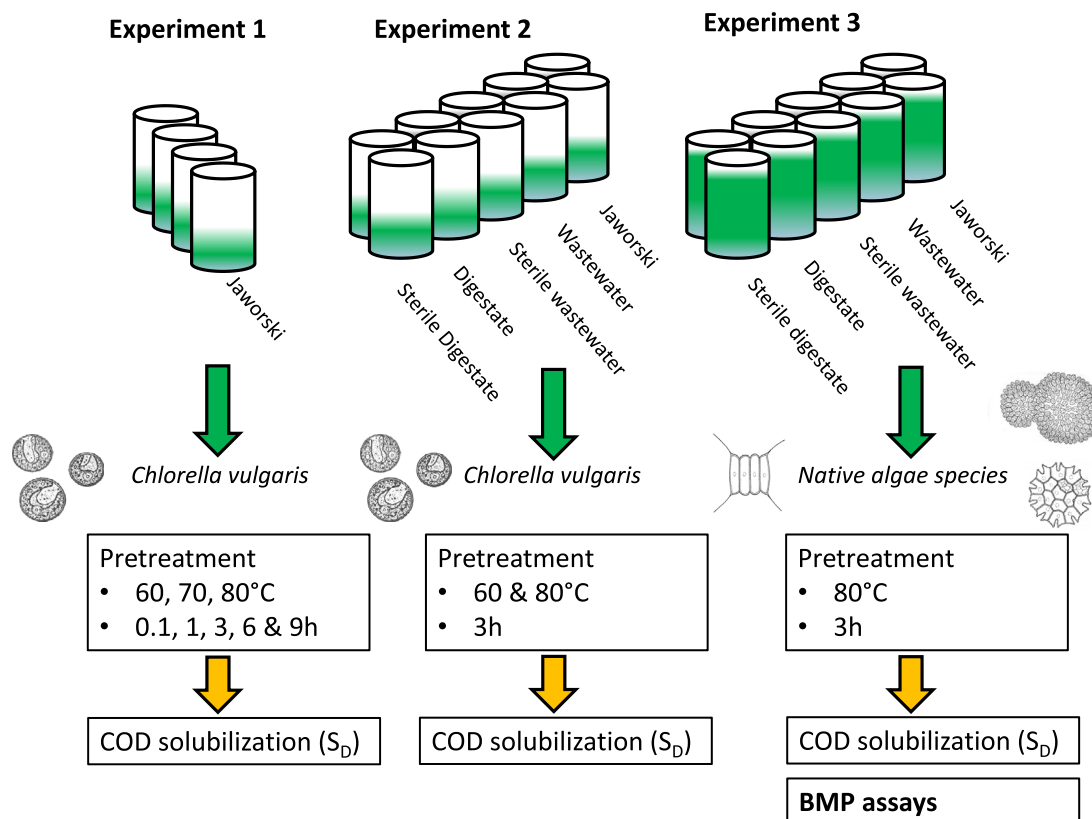
Fig. 1. The experimental setup. The experimental design showing the growth media and pretreatments in each experimental setup. The volume of algae cultivation in each flask was 0.5 L in experiments 1 and 2, and 0.7 L in experiment 3.

Table 1
Characteristics of nutrient media, biomass productivity and nutrient uptake of microalgae. Characteristics of nutrient media, biomass productivity (total volatile solids, VSS (average, range from duplicates in parentheses)), total nitrogen and total phosphorus removal during 8 day batch cultivation and the species of microalgae present in the culture at the end of the cultivation. Dominant species in bolded font.

Experiment/medium	N_{tot} (mg L^{-1})	P_{tot} (mg L^{-1})	N removal (%)	P removal (%)	VSS (g L^{-1})	Algae species
Experiment 1						
Jaworski	16.9	6.4	78	85	0.312 (0.285–0.350)	***Chlorella vulgaris***
Experiment 2						
Jaworski	15.7	6.8	79	88	0.185 (0.183–0.187)	***Chlorella vulgaris***
Wastewater	40.8	3.4	65	79	0.197 (0.157–0.237)	***Chlorella vulgaris;*** *Scenedesmus sp.*
Sterile wastewater	20.8	0.9	50	80	0.207 (0.203–0.207)	***Chlorella vulgaris***
Digestate	125.2	0.5	n.a	n.a	0.198 (0.197–0.200)	***Chlorella vulgaris***
Sterile digestate	73.4	0.3	n.a	n.a	0.147 (0.133–0.153)	***Chlorella vulgaris***
Experiment 3						
Jaworski	15.7	6.7	88	98	0.850 (0.800–0.900)	***Scenedesmus sp., Coelastrum sp.***
Wastewater	43.5	3.4	94	96	1.000 (0.900–1.100)	***Scenedesmus sp., Coelastrum sp.***
Sterile wastewater	29.5	0.9	81	76	1.100 (1.000–1.200)	***Scenedesmus sp., Coelastrum sp.***
Digestate	114	0.3	77	89	0.170 (0.150–0.190)	***Scenedesmus sp.***
Sterile digestate	69.7	0.1	80	51	0.190 (0.160–0.220)	***Scenedesmus sp.*** *Coelastrum sp.*

n.a. not analyzed.

200 μmol photons m^{-2} s^{-1}. The air in- and outflows were filtered with a 0.20 μm filter to prevent contamination. The culture duration was 8 days in all experiments. For the pretreatment and BMP experiments, the algal biomass was concentrated by centrifuging for 5 min at 3000 rpm.

2.4. Pretreatments

All pretreatments were performed in three parallel, closed 50 mL plastic tubes with a liquid volume of about 40 mL. The temperature was increased to the target level (±2 °C) in a water bath

within 5–10 min for 60 and 80 °C, respectively, and subsequently the tubes were kept in an incubator for the specific time for each experiment. The incubation time was measured from the point when the biomass reached the set temperature. After pretreatment, the biomass was cooled at room temperature for about 2 h, and subsequently a sample for COD analysis was taken.

2.5. BMP assays

The BMP was determined in triplicate batch assays using 60 mL glass bottles at mesophilic temperature (35 °C), lasting 46 days

(BMP reported from day 33). Inoculum (15 mL) and substrate were added to the bottles, using a VS$_{substrate}$:VS$_{inoculum}$ ratio of 0.2, recommended for microalgal biomass by Wieczorek et al. (2015). Distilled water was added to make a total liquid volume of 30 mL, and 4 g L^{-1} NaHCO$_3$ was added as buffer. Inoculum alone with distilled water was assayed, and the methane produced was subtracted from that of the substrates. The bottles were flushed for 1 min with nitrogen gas and sealed gas tight with rubber caps and aluminium seals. The methane concentration was measured 1–3 times (3 times during fast methane production phase) per week using gas chromatogram equipped with flame ionizing detector (details in Kinnunen et al. (2015)). The bottles were manually shaken before each measurement. Inoculum was obtained from a full-scale mesophilic anaerobic digester, digesting mixed sewage sludge from the aerobic municipal wastewater treatment process (Viinikanlahti, Tampere, Finland). The inoculum was kept in an open canister at 35 °C for one week prior experiments to activate methanogenic micro-organisms and to reduce residual degradable material. The BMP (33 d) is given under standard conditions (0 °C, 10^5 Pa).

2.6. Analyses and calculations

Total suspended solids (TSS), volatile suspended solids (VSS), total solids (TS), volatile solids (VS) (APHA 2540), and COD (APHA 5220 D) were analyzed according standard methods. Total phosphorus and total nitrogen were analyzed with colorimetric cuvette test (Hach Lange). Soluble COD (sCOD), nitrogen and phosphorus were measured after filtration through a 0.45 μm membrane filter. Volatile fatty acids (VFAs) were measured after filtering through a 0.20 μm filter as described in Kinnunen et al. (2015), and pH was measured using a TPS WP-81 pH meter. Samples of the microalgae cultures were examined under a microscope.

Statistical analysis of the results from BMP assays were done using IBM SPSS software (version 23). A one-way analysis of variance test followed by post hoc multiple comparison (Tukey HSD test) was conducted using 5% significance level after confirming normality (Kolmogorov-Smirnov test) and homogeneity of variances (Levene test).

The degree of solubilization (S$_D$) of the biomass after the pretreatment was calculated using Eq. (1) as in Donoso-Bravo et al. (2011):

$$S_D = \frac{sCOD - s_0COD}{tCOD - s_0COD} * 100,$$
(1)

where the sCOD is the soluble COD after pretreatment, s$_0$COD is the soluble COD of the untreated algae and tCOD is the total COD of the algal biomass.

3. Results and discussion

3.1. Microalgal biomass

Chlorella and native, mixed culture microalgae, cultivated in sterilized or non-sterilized wastewater media, was investigated. The biomass concentrations, nutrient removal efficiency and dominant species of microalgae at the end of each cultivation are shown in Table 1. *Chlorella* was the only species of microalga found after cultivation in Jaworski's medium (experiments 1 and 2) and sterilized wastewater (experiment 2). When non-sterilized wastewater was used as the growth medium (experiment 2), other Chlorophyta species were detected, but *Chlorella* remained the dominant species. When native microalgae from freshwater were cultivated (experiment 3), several species of algae were found in all samples. The most abundant species were in the genera *Scenedesmus* sp. and *Coelastrum* sp. when the algae were grown in the

digestate. *Scenedesmus* sp. formed the majority of the algal population. In the digestate, the *Scenedesmus* sp. cells were smaller (<8 μm) compared with the cultures in other media (⩾8 μm).

Autoclave sterilization changed the nutrient concentrations of the growth media; the nitrogen concentration decreased by 32–49% due to the evaporation of ammonia, while the phosphorus concentration (measured in the filtered samples) decreased 46–76% likely because of precipitation. Marjakangas et al. (2015) found autoclave sterilization decreased nutrient concentrations by 18% for total nitrogen and by 72% for phosphate when anaerobically treated pig wastewater was used.

During the 8 d cultivation, *Chlorella* removed 78–79% of the nitrogen and 85–88% of the phosphorus from Jaworski's medium. Native algae removed 81–94% of the nitrogen and 76–96% of the phosphorus from the wastewater and sterilized wastewater, while less nitrogen and phosphorus were removed from digestate, 77–80% and 51–89%, respectively.

Although the microalgal cultivation conditions were not optimized in this study, the results show that species of native algae grow in wastewater and digestate. Furthermore, the growth was comparable when the algae were cultivated in sterilized or non-sterilized media indicating that bacteria did not necessarily affect growth, supporting a hypothesis recently presented by Park et al. (2015). The same dominant species of native alga was found in all cultivation media. However, the composition of the biomass may differ because of varying media characteristics (e.g., nitrogen and phosphorus concentrations). For example, nitrogen deficiency is known to lead to lipid accumulation in algae, although contradictory results have been found (Marjakangas et al., 2015). Autoclave sterilization reduced the nutrient content of the growth media, which could also increase lipid accumulation. Sterilization of nutrient media (heat or microfiltration) has been frequently used in laboratory studies (Wu et al., 2014), but it is unlikely that autoclaving could be applied at full scale due to the high costs and energy demand.

3.2. The effect of low-temperature pretreatment on COD solubilization

The effect of the pretreatment temperature and duration on COD solubilization was investigated first with the biomass of *Chlorella*, grown in Jaworski's medium (experiment 1, Fig. 2). Pure culture of *Chlorella* was chosen to test the pretreatment temperature and duration as this species is known to have a robust, cellulosic cell wall that is difficult to degrade (Lü et al., 2013). Increased solubilization has been shown to correlate well with increased methane production (Passos et al., 2015). The present results show increase in the S$_D$ (from 1.8 to 10.5%) with increasing treatment time (0–9 h) at all pretreatment temperatures (60, 70 and 80 °C). Higher temperature increased the solubilization more as the S$_D$ at 80 °C was 19–28% and 5–17% more efficient than the treatments at 60 and 70 °C, respectively (Fig. 2).

The effect of the 3 h pretreatment at 60 and 80 °C for COD solubilization of *Chlorella* grown in Jaworski's medium and wastewater media (sterilized and non-sterilized) was investigated to understand the possible role of bacteria in growth media to the solubilization of pure algal culture (experiment 2, Table 2). The solubilization (S$_D$ 6%) of *Chlorella* after pretreatment (at 80 °C for 3 h) was similar to the result in experiment 1 (7%) when the algae were cultivated in Jaworski's medium. Interestingly, the S$_D$ was up to two times higher (10–12%) when *Chlorella* was grown in wastewater or digestate media. However, no difference was observed when *Chlorella* was grown in sterilized and non-sterilized wastewater media. The S$_D$ of *Chlorella* after biological pretreatment (3 h at 60 °C) was 5% for Jaworski's grown biomass. As in the low-temperature pretreatment, in the biological pretreatment, the S$_D$ was higher (10%) for *Chlorella* grown in the wastewater media.
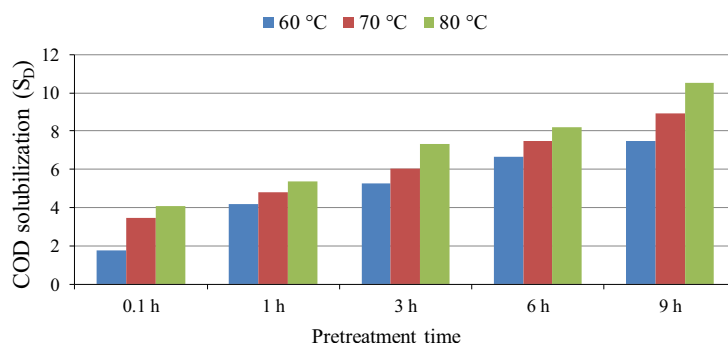
Fig. 2. Pretreatment effects on solubilization of *Chlorella*. The effects of the pretreatment temperature and time on S_D with *Chlorella* cultivated in Jaworski's medium (experiment 1).

Table 2
The pretreatment effects on solubilization of native algae and *Chlorella*. S_D after 3 h low-temperature pretreatment at 60 and 80 °C for *Chlorella* (experiments 1 and 2) and native species of microalgae (experiment 3) cultivated in different nutrient media. Total volatile fatty acids after pretreatment of native microalgae.

	S_D (%) (*Chlorella*) 60 °C	S_D (%) (*Chlorella*) 80 °C	S_D (%) (native microalgae) 80 °C	tVFAs (mg L^{-1}) (native microalgae) 80 °C
Jaworski	5	7	13	0
Wastewater	10	11	12	37
Sterile wastewater	10	12	11	18
Digestate	n.d.	9	10	42
Sterile digestate	n.d.	9	11	40

Again, no difference between sterilized and non-sterilized wastewater was observed (Table 2). This indicate that the increased solubilization of *Chlorella* in low-thermal pretreatments could be related more to different cell composition due different growth media (e.g. Jaworski and wastewater) than to the bacteria in the growth medium.

Use of native algae species is aimed for full-scale applications, and they were investigated in experiment 3, although the seed culture contained also bacteria (based on microscopy). The same level of solubilization after the low-temperature pretreatment (80 °C for 3 h) was observed (S_D 10–13%) no matter in which growth media the algae were cultivated. The S_D of algae in this study was comparable to that observed in previous studies; Alzate et al. (2012) found a S_D of 9–11% after 12–24 h at 55 °C for the biomass dominated by *Scenedesmus*, Cho et al. (2013) reported S_D of only 1.5% for *Scenedesmus* and *Chlorella* (cultivated in synthetic media) biomass after 30 min pretreatment at 50 °C but S_D of 16.9% when temperature was 80 °C. The latter being higher than about 5% (after 30 min) in this study. On the other hand, González-Fernández et al. (2012a) found similar S_D compared to the present study; about 3–5% (estimated from presented data) after 0.5 h pretreatment at 70 and 80 °C, also for *Scenedesmus* biomass.

Although no difference in the S_D was observed for the native algal biomass between different growth media, VFAs were not detected at all after pretreatment of the algae grown in Jaworski's medium, while the VFA concentrations were 18–42 mg L^{-1} (<5% of sCOD) after the pretreatment of the algae grown in wastewater media (Table 2). This could indicate biological hydrolysis and acids formation during the pretreatment and the presence of bacteria in the sterilized wastewater media. González-Fernández et al. (2012b) have reported about 80 mg L^{-1} VFA concentrations after pretreatment at 70 and 90 °C but Lü et al. (2013) found a higher VFA concentration (457 mg L^{-1} as carbon) at the early stage of AD of *Chlorella* when the biomass of *Chlorella* was bioaugmented with anaerobic bacteria *Clostridium thermocellum*. However, Passos et al. (2016) did not detect VFAs from microalgae grown in wastewater after pretreatment similar to the pretreatment in the present study (2 h at 80 °C).

3.3. Biomethane potential

The BMP for the native microalgal biomass cultivated in Jaworski's media, non-sterilized and sterilized wastewater and non-sterilized and sterilized digestate from AD of pulp and paper biosludge was investigated with and without pretreatment (Fig. 3 and Table 3). The BMP for the native microalgae was 222–252 L CH_4 kg^{-1} VS for the biomass grown in the Jaworski and non-sterilized and sterilized wastewater media. Statistically significant (p = 0.05) difference was found between BMPs for algae grown in Jaworski and wastewater but not between sterilized and non-sterilized wastewater. The BMP for the biomass grown in the digestate and the sterilized digestate was clearly lower, 154–182 L CH_4 kg^{-1} VS, the difference being statistically significant compared with other growth media, but also between sterilized and non-sterilized digestate. Methane production started immediately for all biomass samples, and 70–90% of the methane was formed within 10 days from the beginning of the assay.

Varying BMPs between 107 and 410 L CH_4 kg^{-1} VS have been found for *Scenedesmus* dominating biomass, mainly cultivated in different synthetic media (Frigon et al., 2013; Mendez et al., 2014; Zhen et al., 2016; Roberts et al., 2016), and only results reported by Frigon et al. (2013) exceeding BMP of 300 L CH_4 kg^{-1} VS. The authors reported *Scenedesmus* sp. has a BMP of 306 L CH_4 kg^{-1} VS when grown in synthetic medium but clearly higher, 410 L CH_4 kg^{-1} VS, when grown in wastewater. The result is contradictory to findings in the present study, where algae biomass had higher BMP when cultivated in Jaworski medium, compared with wastewater. No difference between the BMP of algae grown in sterilized and non-sterilized wastewater was found. The clearly lower BMP of digestate grown algae in the present study could be explained by the different cell composition due to the different growth media, rather than bacteria biomass from waste originated growth media.

Pretreatment (3 h at 80 °C) increased the BMPs for all microalgae samples by 11–24%. The increase was statistically significant (p = 0.05) for all samples, except digestate grown algae biomass (Table 3). The highest increase in the BMP was for algae grown
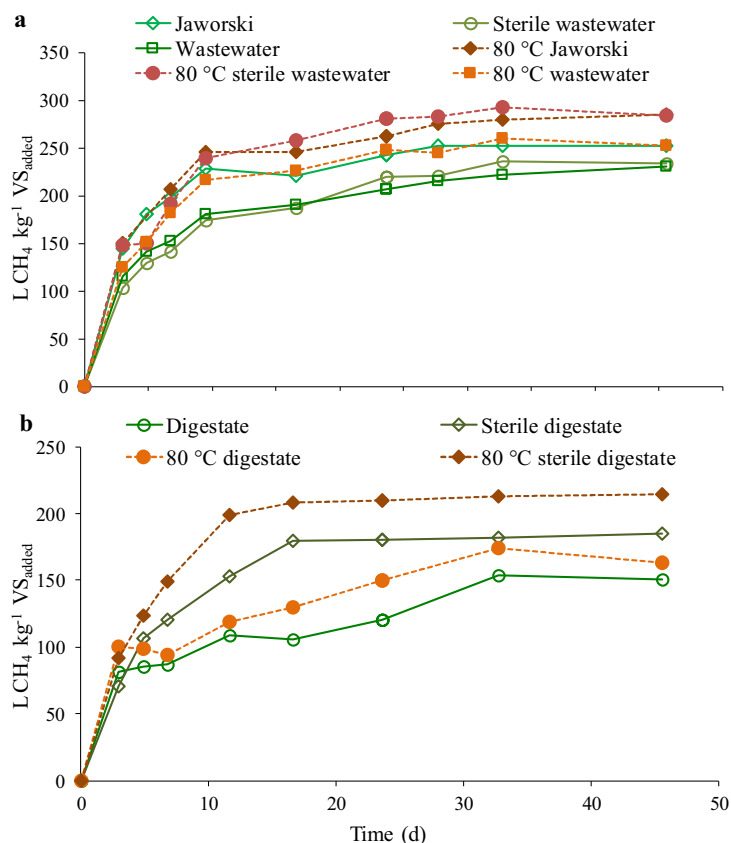
Fig. 3. Cumulative methane production from microalgae. Methane production in batch assays from native algae grown in (a) synthetic media and sterilized or non-sterilized wastewater (b) in the sterilized or non-sterilized centrate of digested pulp and paper biosludge. All results are from experiment 3.

Table 3
BMP for untreated and pretreated native microalgae. BMP for algal biomass in this work. Pretreatment efficiency means the percent change in the BMP between the untreated and pretreated biomass.

Dominant species	Medium	BMP untreated (L CH$_4$ kgVS^{-1})	BMP pretreated (L CH$_4$ kgVS^{-1})	Pretreatment efficiency (%)
Scenedesmus sp., Coelastrum sp.	Synthetic (Jaworski)[a]	252 (3)[b,d,e]	280 (9)[f]	+11
Scenedesmus sp., Coelastrum sp.	Wastewater[b]	222 (10)[a,d,e]	259 (13)[f]	+17
Scenedesmus sp., Coelastrum sp.	Sterile wastewater[c]	236 (2)[d,e]	292 (7)[f]	+24
Scenedesmus sp.	Digestate[d]	154 (5)[a,b,c,e]	173 (10)	+12
Scenedesmus sp.	Sterile digestate[e]	182 (3)[a,b,c,d]	213 (6)[f]	+17

[a,b,c,d,e]Indicates significantly different BMP between algae biomass grown in specific media (marked in medium column), marked with superscript (p = 0.05).
[f]Indicates BMP after pretreatment significantly different compared with untreated (p = 0.05).

in sterilized wastewater, having also the highest BMP (292 L CH$_4$ kg^{-1} VS) observed in this study.

The impact of the pretreatments on the BMP of native algae (mainly *Scenedesmus*) was comparable with that observed in previous studies, although the results vary widely again. González-Fernández et al. (2012a) found pretreatment at 70 °C to improve the BMP of *Scenedesmus* by 9% and the pretreatment at 80 °C by 57%, latter being clearly higher improvement than in this study. González-Fernández et al. (2012a, 2012b) also showed that at about 80 °C is a threshold temperature, where the *Scenedesmus* cell wall is damaged, while at lower pretreatment temperature the increased solubility and BMP are likely due exopolymers. Mendez et al. (2014) found 40 min pretreatment at 120 °C to improve the BMP of *Scenedesmus* by 21–27%, about the same extent than this study but at higher temperature.

The results in the present study suggests that the methane production and the effect of low-temperature pretreatment on algae degradability are not affected whether the algae cultivation media is sterilized or non-sterilized. Instead, the present results indicate that the differences in the BMP might originate from the different characteristics of the algal biomass caused by different composition of growth media. However, the impact of bacteria on AD of microalgae may be species specific (both algae and bacteria), and further research is needed, where identification of microorganisms in microalgae/bacteria biomass is conducted.

4. Conclusions

The BMP of the untreated native boreal microalgae (mainly *Scenedesmus* sp.) was 154–252 L CH$_4$ kg^{-1} VS and was increased with low-temperature (3 h at 80 °C) pretreatment by 11–24%. No differences in BMP or S$_D$ was obtained between algal biomass grown in sterilized or non-sterilized wastewater media. The differences in the BMP and solubilization during the pretreatment might originate from the different compositions of the algal biomass, following cultivation in different growth media, rather than from the presence of bacteria in cultivation medium.

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