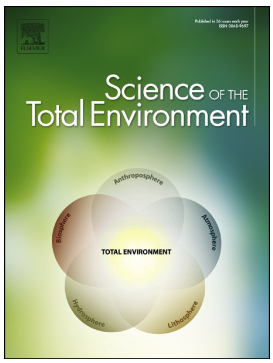

The capture technology matters: Composition of municipal wastewater solids drives complexity of microbial community structure and volatile fatty acid profile during anaerobic fermentation

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The capture technology matters: Composition of municipal wastewater solids drives complexity of microbial community structure and volatile fatty acid profile during anaerobic fermentation

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Abstract

The production of volatile fatty acids (VFAs) represents a relevant option to valorize municipal wastewater (MWW). In this context, different capture technologies can be used to recover organic carbon from wastewater in form of solids, while pre-treatment of those solids has the potential to increase VFA production during subsequent fermentation. Our study investigates how VFA composition produced by fermentation is influenced (i) by the choice of the capture technology, as well as (ii) by the use of thermal alkaline pre-treatment (TAP). Therefore, the fermentation of solids originating from a primary settler, a micro-sieve, and a high-rate activated sludge (HRAS) system was investigated in **continuous lab-scale fermenters, with and without TAP.** Our study demonstrates that the capture technology strongly influences the composition of the produced solids, which in turn drives the complexity of the fermenter's microbial community and ultimately, of the VFA composition. Solids captured with the primary settler or micro-sieve consisted primarily of polysaccharides, and led to the establishment of a microbial community specialized in the degradation of complex carbohydrates. The produced VFA composition was relatively simple, with acetate and propionate accounting for >90% of the VFAs. In contrast, the HRAS system produced biomass-rich solids associated with higher protein contents. The microbial community which then developed in the fermenter was therefor more diversified and capable of converting a wider range of substrates (polysaccharides, proteins, amino acids). Ultimately, the produced VFA composition was more complex, with equal fractions of iso-acids and propionate (both ~20%), while acetate remained the dominant acid (~50%). Finally, TAP did not significantly modify the VFA composition while increasing VFA yields on HRAS and sieved material by 35% and 20%, respectively. Overall, we demonstrated that the selection of the technology used to capture organic substrates from MWW governs the composition of the VFA cocktail, ultimately with implications for their further utilization.

Keywords

Volatile fatty acids; Fermentation of municipal wastewater solids; Micro-sieve; High-rate activated sludge; Thermal-alkaline pre-treatment; Anaerobic microbial community structure.

1 Introduction

The anaerobic treatment of municipal wastewater (MWW) can be advantageous compared to aerobic treatment, in terms of net energy balance and needs for the handling of biosolids (McCarty, 2018). For this purpose, organic carbon must first be efficiently captured before being converted into methane *via* anaerobic digestion (McCarty et al., 2011), or into volatile fatty acids (VFAs) *via* fermentation (Kleerebezem et al., 2015). VFAs are relevant products for direct use in the pharmaceutical, chemical and food industries (Atasoy et al., 2018). VFAs can also be upgraded to valuable end-products such as polyhydroxyalkanoates (PHA), a precursor of bioplastics (Alloul et al., 2018). The properties and subsequent industrial applications of the PHA polymers depend on the VFA composition (Bengtsson et al., 2010; Lemos et al., 2006). It is hence crucial to predict and control the VFA composition that results from the fermentation of the captured organic carbon. But carbon capture from MWW can be performed using different technologies, each relying on distinct mechanisms: sedimentation for primary settlers, filtration for micro-sieves or combined microbial growth and bio-sorption in high-rate activated sludge (HRAS) systems. While existing wastewater treatment plants (WWTPs) mainly rely on primary settlers, water resource recovery facilities (WRRFs) might use micro-sieves combined with HRAS systems in an attempt to capture all the organic carbon from MWW. Since the choice of technology determines the capture mechanism, it is key to verify the existence of a link between the capture technology, the composition of the captured organic carbon and the VFA composition in the fermenter. Ultimately, we must understand how the technological transition, away from the primary settler towards micro-sieves and HRAS systems, could impact VFA production.

Maximising the amount of organic carbon directed towards anaerobic treatment requires to capture both the particulate and the soluble fractions. Primary settlers capture 40-60% of the solids contained in MWW (Tchobanoglous et al., 2013). But primary settlers have a large footprint, and more compact pre-treatment technologies are being developed. Micro-sieves (e.g., drum-screens) achieve similar performances to primary settlers in terms of solids capture but require much less space. Micro-sieves also help to capture higher fractions of biodegradable organics, such as cellulose (Bahreini et al., 2020; Schmidt and Schubert, 2018). Both primary settlers and micro-sieves capture organic carbon in the

particulate form, associated with high polysaccharide contents (Da Ros et al., 2020; Elefsiniotis and Oldham, 1994). However, dissolved organic carbon can represent up to 50% of the total organic carbon in MWW (Levine et al., 1991) and must be captured with an appropriate technology. HRAS systems have been widely applied on full-scale WWTPs; such systems allow capturing dissolved organics in form of bacterial biomass, and particulate organics *via* adsorption onto the flocs (Rahman et al., 2019; Sancho et al., 2019; Wett et al., 2020). Further, the low solid retention times (SRT) (<1 day) applied to HRAS systems allow minimizing mineralisation and losses of organics (Jimenez et al., 2015). Since HRAS systems promote microbial growth, the produced solids intuitively contain higher proportions of proteins. If transitioning from WWTPs to WRRFs requires replacing the state-of-the-art primary settler by a combination of micro-sieve and HRAS system, it is important to better understand to what extent sieved-material and HRAS differ from conventional primary sludge in terms of composition, and thus, what the implications are for the VFA composition produced during fermentation.

The fermentation of solids from primary settlers has been widely investigated and mostly aimed at improving the performance of enhanced biological phosphorus removal systems. VFA-rich effluents (70 – 100% of the soluble chemical oxygen demand (COD)) were successfully produced and consisted of acetic and propionic acids mainly (Ahn and Speece, 2006; Elefsiniotis and Oldham, 1994; Ucisik and Henze, 2008). However, the proportion of acetic to propionic acid greatly varies depending on operating conditions (Ahn and Speece, 2006; Pittmann and Steinmetz, 2013) and on the MWW composition (Ucisik and Henze, 2008). In place of primary settlers, the WWTP of the future might rely on micro-sieves combined with HRAS systems. Yet, only few studies investigated the production of VFAs from sieved material and HRAS. Bahreini et al. (2020) compared the fermentation of primary sludge and sieved material originating from the same MWW source: VFA compositions were similar, with acetate as the predominant compound, followed by propionate and butyrate. On the contrary, Da Ros et al. (2020) observed a VFA composition dominated by propionate (52%) over acetate (30%) when fermenting sieved material. During the fermentation of HRAS, strong variations of the acetate fraction (56-89%) at the expense of propionate, butyrate, and/or iso-valerate were observed depending on operating temperature and pH (Cagnetta et al., 2016). A comparison of the results from the

aforementioned studies is very difficult since operating conditions, reactor operation mode and MWW sources differ from study to study. To date, no study has systematically investigated the fermentation of primary sludge *vs.* sieved material and HRAS under similar operating conditions and using a single MWW source. Consequently, the specific influence of the different capture technologies (primary settler vs. micro-sieve and/or HRAS system) on the VFA composition remains unclear.

Microbial hydrolysis of solids is a slow process and the rate-limiting step of acidogenic fermentation (Eastman and Ferguson, 1981). Pre-treatment of the solids is often applied to increase solubilisation and, ultimately, to maximise the solids to VFA conversion yield. Approaches such as high-pressure thermal hydrolysis, low-temperature alkaline pre-treatment (TAP) and enzymatic treatment, have been successfully used for the solubilisation of primary sludge, waste activated sludge (WAS) and sieved material (Bahreini et al., 2020; Morgan-Sagastume et al., 2011; Nazari et al., 2017; Pang et al., 2020; Tan et al., 2012). Out of the different pre-treatment approaches, TAP seems particularly promising. TAP is cheaper and easier to operate compared to high-pressure thermal hydrolysis (Toutian et al., 2021). Also, TAP helps solubilising a wide range of solids (Nazari et al., 2017), while enzymatic treatment is by nature compound-specific (e.g., cellulase or protease) (Bahreini et al., 2020; Pang et al., 2020). While most efforts in recent years have focused on increasing VFA yields, little emphasis has been put on comprehending to what extent pre-treatment of solids affects the VFA composition in the fermenter (Liang et al., 2021). Tan et al. (2012) observed a similar VFA composition during the fermentation of WAS with and without TAP (60°C, pH 11). However, the batches were inoculated with the same seeding sludge that had not undergone TAP. Yet, thermal pre-treatment of solids has been shown to cause drastic changes in the microbial communities that develop in full-scale digesters (Mei et al., 2017). To date, it remains unclear to what extent TAP of solids influences the microbial community structure in fermenters, and the composition of the VFAs in turn produced.

Ultimately, the type of VFAs produced depends on the microbial communities growing on the organic carbon substrate. For instance, Rico et al. (2021) observed that fermentation of the same cellulose feedstock by three different microbial inocula resulted in three distinct VFA spectra. Detailed analysis of the microbial communities is therefore a relevant additional tool to explain why the VFA

composition in a fermenter may change as a function of the influent solids composition or due to pre-treatment of the solids.

Our study aims (i) to identify the link between the choice of the capture technology, the composition of the resulting solids and ultimately, the VFA composition, and (ii) to evaluate to what extent TAP modifies the VFA composition while increasing the solubilisation efficiency. We therefore investigated the solubilisation and especially fermentation of solids originating from a primary settler, a micro-sieve and a HRAS system, with and without TAP in continuous lab-scale systems. The characteristics of influent solids (nitrogen and phosphorus content, morphology, visual nucleic acid abundance), overall system performances (effluent VFA composition, solubilisation of solids, solids to VFA conversion yields) and microbial community structures were thus monitored over several months.

2 Materials and methods

2.1 Experimental approach and harvesting of solids

Primary sludge, sieved materials and HRAS were harvested from the Eawag pilot-scale WWTP, which is connected to a combined sewer system (Dübendorf, Switzerland). Primary sludge originated from the primary clarifier (hydraulic retention time (HRT) of 1 hour). Sieved material was collected with a pilot-scale drum-screen (model LIQUID Mini, HUBER, Germany). Excess sludge was collected from a HRAS system (SRT of ~0.5 days, dissolved oxygen of 0.8 mg L^{-1}) treating the effluent of the primary clarifier.

Two sets of fermentation experiments were conducted: with and without TAP. Experiments without TAP were performed on primary sludge, sieved material and HRAS while experiments with TAP were conducted on sieved materials and HRAS only. Since the composition of primary sludge and sieved material were very similar, TAP of primary sludge was not explicitly investigated. The experimental system without TAP consisted of a 8.4 L fermenter followed by a 9.5 L clarifer. A pre-treatment unit was added upfront the fermenter for the system with TAP (**Figure 1**). In the HRAS experiment

without TAP, solid-liquid separation in the clarifier was more challenging, so a second run was performed to confirm our results (Run1 and Run2). To ensure a similar organic loading in all experiments, the concentration of the freshly harvested solids was adjusted to 2.5 gCOD L^{-1}. Primary sludge and sieved material were therefor diluted with tap water while HRAS was concentrated *via* sedimentation. The adjusted influents were stored at 4°C and renewed on a weekly basis.

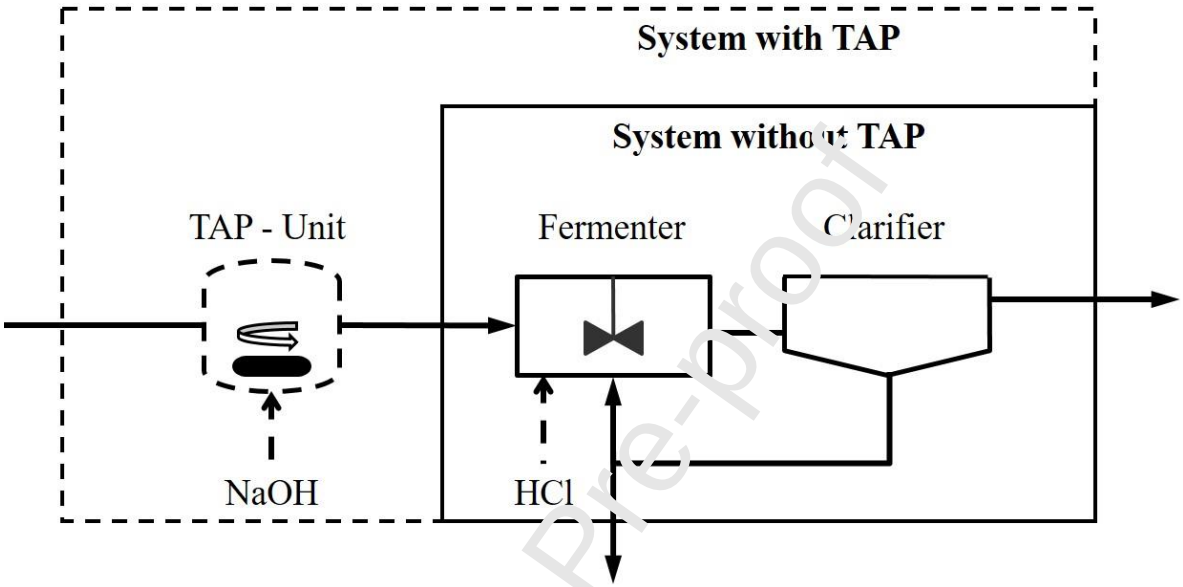

Figure 1: Schematics of experimental systems without TAP (solid lines only) and with TAP (solid lines + dashed lines).

2.2 Operating conditions and detailed experimental set-up

The duration of the individual experiments varied between 56 - 75 days (**Table 1**). Temperature in the fermenters was controlled at 20 – 22 °C (Elefsiniotis and Oldham, 1994). The SRT of the biological systems (fermenter and clarifier) was controlled between 14 - 16 days (19 days in the case of sieved material without TAP) to avoid growth of methanogens (**Table 1**) (Elefsiniotis and Oldham, 1994). No inhibitors of methanogenesis were used in this study. The HRT in the fermenter was 14 hours, resulting in organic loadings of 4.2 – 4.8 gCOD L^{-1} day^{-1} (**Table 1**), in the same order of magnitude than loads reported in literature (Da Ros et al., 2020; Ucisik and Henze, 2008; Yuan et al., 2009). The TAP unit was operated at 60°C and at pH = 11 (Rani et al., 2012; Tan et al., 2012). pH was controlled through automated addition of 2M NaOH solution. Preliminary batch tests on sieved material and HRAS suggested an optimal HRT of 2 hours (**SI Figure A.1**). Accordingly, the TAP unit had a

working volume of 1.2 L. The decoupling of SRT and HRT allowed to maintain a neutral pH in the fermenters during the experiments without TAP (pH 6.5 - 7.0). In the experiment with TAP, the pH in the fermenter was controlled at pH 7 through addition of a 2M HCl solution. All fermenters and TAP units were jacketed and equipped with temperature and pH sensors (Endress & Hauser, Switzerland). Sensors were connected to a programmable logic controller (PLC) and monitored by a supervisory control and data acquisition (SCADA) system. Mechanical and magnetic stirrers were used for the mixing of the fermenters and TAP units, respectively.

***Table 1**: Key operating conditions and exact duration of the different experiments. For organic loading and SRT: Mean values ± standard deviation and the number of measurements (n).*

		Primary Sludge	**Sieved Material**		**HRAS**		
Operating Conditions		no TAP	no TAP	with TAP	no TAP (Run1)	no TAP (Run2)	with TAP
Organic loading	[gCOD L^{-1} day^{-1}]	4.8 ± 1.4 (15)	4.2 ± 1.2 (15)	4.5 ± 1.4 (13)	4.1 ± 0.8 (17)	4.1 ± 1.1 (13)	4.1 ± 0.9 (14)
System SRT	[days]	14 ± 2 (15)	19 ± 2 (15)	14 ± 0 (14)	14 ± 3 (5)	16 ± 2 (10)	14 ± 0 (14)
Experiment duration	[days]	75	75	60	75	56	60

2.3 Analytical methods

2.3.1 Chemical analyses and suspended solids measurements

Samples were taken twice a week from the influent and effluent of the fermenter (and TAP unit). Total COD, total nitrogen (TN), ammonium nitrogen (NH_4-N), total phosphorus (TP) and ortho-phosphate (PO_4-P) in samples were directly analyzed with colometric assays (Hach-Lange, Germany, LCK 014, 114, 303, 304, 338, 349, 350). To increase the accuracy of total COD, TN and TP measurements, samples were mechanically homogenized (T50 ULTRA-TURRAX, IKA, Germany) for two minutes at 16'000 rpm. Soluble COD (sCOD), NH_4-N and PO_4-P were measured after filtration at 0.45 µm (Macherey Nagel, Nanoclor Chromafil membranefilter GF/PET 0.45 µm, Germany). Particulate COD (pCOD) was calculated by subtracting the measured sCOD from the measured total COD. VFAs, namely acetate, propionate, iso-butyrate, butyrate, iso-valerate and valerate, were measured via headspace solid-phase microextraction (HS-SPME) followed by chromatography coupled to a flame ionization detector (GC-FID) (Trace 1300 GC, Thermo Scientific, USA) (Feng et al., 2008). Prior to VFA analysis, 3 g of NaCl were added to 20 mL of filtered sample (0.45 µm), whereafter pH was adjusted to 1 – 2 by addition of HCl. Total suspended solids (TSS) and volatile suspended solids (VSS) were measured according to standard methods (APHA, 2012). TSS was additionally measured in the clarifier (mixed before sampling) in order to properly control the SRT in the system.

2.3.2 Stereo- and epifluorescence microscopy

The morphology of the influent solids was observed via stereomicroscopy using a SZX10 microscope equipped with a DP72 camera in order to evaluate the presence of cellulose fibers (both Olympus, Japan). The biomass present in the different influent solids was visualized by epifluorescence microscopy (Leica, DMI 6000B, Germany). Samples were incubated in the dark for 15 min and at 37°C within a 0.2 mM (final) SYBR® Green I stain solution (ThermoFisher Scientific, USA). Immediately after, pictures were taken in bright-field mode (DFC 350 FXR2) as well as fluorescence mode (GFP – filter, DFC 295) and combined to a multi-chanel picture using the LAS AF software (all

Leica, Germany). For sieved material and HRAS samples, pictures were taken before and after TAP. For consistency, same gain and exposure settings were used in fluorescence mode.

2.3.3 **Adenosin tri-phosphate (ATP) measurements**

The total ATP content of freshly sampled solids (µM $gTSS^{-1}$) was periodically quantified using the BacTiter-Glo™ Microbial Cell Viability Assay (Promega Corporation, , USA) and a GloMax® 20/20 Luminometer (Turner BioSystems, USA). 100 µL of sludge sample (diluted with deionized water) were mixed with 100 µL of BacTiter-Glo™ reagent after incubation in the dark at 38°C for 4 minutes. After another 20 seconds of incubation, the relative light units were measured as an integral over 10 seconds and converted to ATP concentrations using a calibration curve (**Supporting Information B**) (Hammes et al., 2010).

2.3.4 **Microbial community analysis**

Samples from the influent (after TAP, when applied) and from the fermenters were collected weekly for 16s rRNA gene sequencing. 1.5 mL of sludge were rinced with an ice-cold phosphate buffered saline (PBS) solution (peletting at 12'000 ×g – resuspension in PBS – peletting at 12'000 ×g). Pellets were resuspended in 3-4 mL of PBS solution, homogenized with a glass homogenizer, and stored at -80°C until DNA extraction. DNA extraction and bacterial 16S rRNA amplicon sequencing was carried out as described in Layer et al. (2019). 200 µL of the homogenized biomass were mixed with 400 µL of TE buffer (10 mM Tris-HCl, 1 mM EDTA-Na_2 pH 8.0) and 100 µL of lysozyme solution (25 mg mL^{-1}), prior to incubation for 1 hour at 37 °C. DNA was extracted using Maxwell® 16 and Tissue DNA purification kits (all Promega, USA) according to the manusfacturer instructions. Quality measurement and quantification of the extracted DNA samples were assessed with agarose gels and fluorometric assays (Qubit ver. 2.0, Life Technologies, USA), respectively. Bacterial 16S rRNA gene hypervariable regions V1–V2 were amplified in a T3000 Thermocycler (Biometra, Germany) using 27F and 338R universal primers with overhang adapters (5'TCGTCGGCAGCGTCAGATGTGTATAAGAGACAG-AGMGTTYGATYMTGGCTCAG3') and

(5'GTCTCGTGGGCTCGGAGATGTGTATAAGAGACA-GGCTGCCTCCCGTAGGAGT3'). Amplification products were quantified on a Fragment Analyzer System with a NGS fragment kit (both Agilent, USA) prior to sequencing at the Lausanne Genomic Technologies Facility (University of Lausanne, Switzerland). Multiplex paired-end sequencing (2x250 bp) was carried out on an Illumina MiSeq platform. The raw sequences are accessible under DOI:10.25678/0003YF (**Eawag Research Data Institutional Collection (ERIC)) (DOI will be unlocked once manuscript is published)**.

The definition of OTU's and the taxonomic affiliation was performed using FROGS pipeline and the generated output is provided in the **Supporting Information B** file (Escudie et al., 2018; Poirier et al., 2018). OTU's containing less than 0.01% of all sequences were excluded. Taxons were affiliated using 16S Silva 138 (Quast et al., 2013). The freeware R version 4.0.2 (R Core Team, 2020) running on RStudio (version 1.3.1093) was used for numerical ecology analysis and inference statistics (Coral et al., 2018). Data clustering and principal component analysis (PCA) were carried out using the Ward method on Hellinger transformed distance matrix using the Vegan package (Oksanen et al., 2020). Vegan package was also used for redundancy analysis, a direct extension of multiple regression to the modelling of multivariate response data. The redundancy analysis forms an ordination of a set of response variables, constrained so that the ordination variables that are formed are linear combinations of a set of explanatory variables (Borcard et al., 2011). Heatmaps were generated using Spearman pairwise correlations between environmental and microbial data sets. Detailed microbial community analysis (PCA, redundancy analysis, correlation heatmaps) was performed at the family taxonomic level. Working at this taxonomic level of detail already allowed to link certain metabolic functions to individual taxa, but at the same time, to minimize the amount of multi- and non-affiliations. In addition, we selectively considered the phylum or genus level whenever it benefited the interpretation, discussion and contextualization of the data.

2.4 Calculation

2.4.1 **Characterisation of influent solids**

One objective of this study was to better understand to what extent the composition of solids is governed by the type of the capture technology. Influent solids were thus characterised in terms of nitrogen and phosphorus content; two parameters indirectly indicating the polysaccharides, proteins and lipids content of the solids. The nitrogen content was calculated as:

$$\text{Nitrogen content}\left[\frac{\text{gN}}{\text{gpCOD}}\right]=\frac{(TN_{in}-NH_4\text{-}N_{in})}{(COD_{in}-sCOD_{in})} \quad (1)$$

with TN_{in} the influent TN (mgN L^{-1}), $NH_4\text{-}N_{in}$ the influent NH_4-N (mgNH_4-N L^{-1}), NO_x-N being negligeable, COD_{in} the total influent COD (mgCOD L^{-1}), and $sCOD_{in}$ the influent sCOD (mgCOD L^{-1}). Similarily, the phosphorus content was calculated as:

$$\text{Phosphorus content}\left[\frac{\text{gP}}{\text{gpCOD}}\right]=\frac{(TP_{in}-PO_4\text{-}P_{in})}{(COD_{in}-sCOD_{in})} \quad (2)$$

with TP_{in} the influent TP (mgP L^{-1}) and $PO_4\text{-}P_{in}$ the influent PO_4-P (mgPO_4-P L^{-1}).

2.4.2 **Solubilisation of influent solids**

For all experiments, the overall observed solubilisation was calculated as:

$$\text{Overall Solubilisation}\left[\frac{\text{gsCOD}}{\text{gpCOD}_{in}}\right]=\frac{(sCOD_{eff}-sCOD_{in})}{(COD_{in}-sCOD_{in})} \quad (3)$$

with $sCOD_{eff}$ the fermenter's effluent sCOD (mgCOD L^{-1}). In the experiments with TAP, we distinguished between the solubilisation in the TAP unit (Solubilisation TAP) and the solubilisation in the fermenter (Solubilisation Fermenter):

$$\text{Solubilisation TAP}\left[\frac{\text{gsCOD}}{\text{gpCOD}_{in}}\right]=\frac{(sCOD_{TAP}-sCOD_{in})}{(COD_{in}-sCOD_{in})} \quad (4)$$

And

$$\text{Solubilisation Fermenter}\left[\frac{\text{gsCOD}}{\text{gpCOD}_{in}}\right]=\text{Overall Solubilisation - Solubilisation TAP} \quad (5)$$

with $sCOD_{TAP}$ the sCOD in the effluent of the TAP unit (mgCOD L^{-1}).

2.4.3 **VFA yield**

This study focused strictly on the conversion of influent solids to VFAs. Therefore, the VFA yield was calculated as:

$$\text{Overall VFA yield}\left[\frac{\text{gCOD}_{\text{VFA}}}{\text{gpCOD}_{\text{in}}}\right] = \frac{(\text{VFA}_{\text{eff}} - \text{sCOD}_{\text{in}})}{(\text{COD}_{\text{in}} - \text{sCOD}_{\text{in}})} \quad (6)$$

with VFA_{eff} the fermenter's effluent VFA concentration (mgCOD L^{-1}). A yield of 0 was considered in case sCOD_{in} exceeded VFA_{eff}. In the experiments with TAP, we specified the respective contribution of the TAP unit and the fermenter to the overall VFA yield as follows:

$$\text{VFA yield TAP}\left[\frac{\text{gCOD}_{\text{VFA}}}{\text{gpCOD}_{\text{in}}}\right] = \frac{(\text{VFA}_{\text{TAP}} - \text{sCOD}_{\text{in}})}{(\text{COD}_{\text{in}} - \text{sCOD}_{\text{in}})} \quad (7)$$

And

$$\text{VFA yield Fermenter}\left[\frac{\text{gCOD}_{\text{VFA}}}{\text{gpCOD}_{\text{in}}}\right] = \text{Overall VFA yield} - \text{VFA yield TAP} \quad (8)$$

with VFA_{TAP} the VFA concentration in the effluent of the TAP unit (mgCOD L^{-1}).

2.4.4 **Acidification degree**

The acidification degree is a measure for the orientation of the fermentation towards VFA production and was calculated as:

$$\text{Acidification degree [-]} = \frac{\text{VFA}_{\text{eff}}}{\text{sCOD}_{\text{eff}}} \quad (9)$$

2.4.5 **Definition of stable-operation**

Average values for solubilisations, VFA yields, and acidification degrees were calculated for the stable operation period only. Stable operation was defined as the time-period, during which individual values did not significantly vary (starting from 20-30 days after start-up). The calculated individual values and time series can be found in the **Supporting Information B** file. The raw data of all

measured parameters is accessible under DOI:10.25678/0003YF (**Eawag Research Data Institutional Collection (ERIC)) (DOI will be unlocked once manuscript is published)**.

3 Results

3.1 How does the capture technology influence the composition of solids?

The characteristics and microscopic observations of the different influent solids are reported in **Table 2** and **Figure 2**, respectively. Our results indicate that the type of capture technology directly governed the composition of the produced solids. The primary settler and the micro-sieve favoured the capture of fibrous solids with low nutrient, nucleic acid and ATP contents, indicative of a low biomass content (**Figure 2a and 2b**). On the contrary, the solids produced by the HRAS-system consisted mainly of floccular biomass associated with high nutrient, nucleic acid and ATP contents (**Figure 2c**).

The organic content (VSS/TSS) was slightly higher for sieved material and primary sludge (>0.90) than for HRAS (<0.90). The nitrogen and phosphorus content were considerably higher for HRAS (>50 mgN $gpCOD^{-1}$ and >9 mgP $gpCOD^{-1}$) than for primary sludge and sieved material (<15 mgN $gpCOD^{-1}$ and <3.5 mgP $gpCOD^{-1}$). Also, the ATP concentrations were ca. 10 times higher in HRAS than for the primary sludge or sieved material. Visual observations of the epifluorescence pictures indicate in addition a higher abundance of nucleic acids for the HRAS, compared to the other solids (**Figure 2f-2h**). After TAP, both HRAS and sieved materials were characterised by a lower nucleic acid signal intensity (**Figure 2i and 2j**). Also, the flocs size of HRAS increased considerably after TAP (**Figure 2e**).

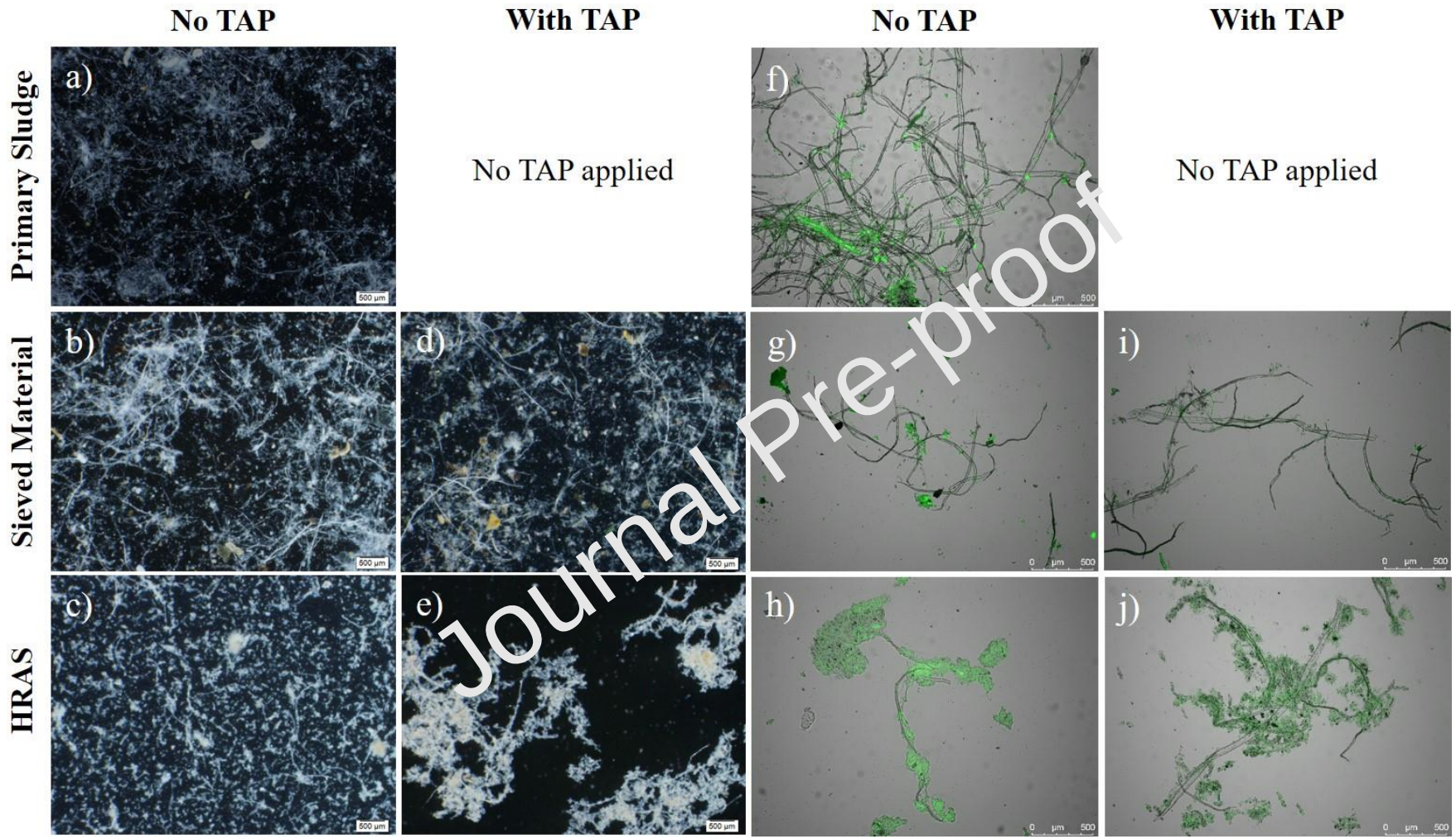

***Figure 2**: Stereo and epifluorescence microscopic pictures before and after pre-treatment (no TAP was applied to primary sludge). Pictures f)-j): Overlay of the brightfield image (in grey) with the fluorescent signal of the SybrGreen stain bound to nucleic acids (in green). Bar scales: 500 µm.*

***Table 2**: Composition of the different solids captured with the primary settler, micro-sieve and HRAS. Mean values ± standard deviation and the number of measurements (n). n/a: not available.*

		Primary Sludge	**Sieved Material**		**HRAS**		
Influent Composition		no TAP	no TAP	with TAP	no TAP (Run1)	no TAP (Run2)	with TAP
VSS/TSS	[-]	0.91 ± 0.06 (15)	0.95 ± 0.04 (15)	0.90 ± 0.05 (18)	0.87 ± 0.04 (17)	0.85 ± 0.02 (12)	0.88 ± 0.04 (19)
Nitrogen content	[mgN gpCOD $^{-1}$]	14.9 ± 3.1 (14)	11.0 ± 2.7 (14)	14.3 ± 3.4 (8)	62.7 ± 9.4 (17)	72.6 ± 9.4 (12)	52.2 ± 11.2 (7)
Phosphorus content	[mgP gpCOD $^{-1}$]	3.3 ± 0.8 (15)	2.4 ± 0.6 (15)	2.9 ± 0.9 (18)	9.5 ± 3.0 (17)	13.5 ± 2.2 (13)	11.5 ± 4.6 (12)
ATP	[µM gTSS^{-1}]	0.15 ± 0.06 (6)	0.05 ± 0.05 (5)	n/a	1.35 ± 0.54 (6)	n/a	n/a

3.2 How does the composition of the influent solids and their pre-treatment influence the microbial community structures in the fermenters?

Microbial communities were monitored in the influent and in the fermenters throughout the experiments (**Figure 3**). The microbial communities were very similar in the primary sludge and sieved material fermenters, but clearly different in the HRAS fermenter, as highlighted by the clustering of the samples on the PCA plot (**Figure 4**). Applying TAP caused a drastic change of the microbial community structure in the fermenters compared to the experiment without TAP (**Figure 4**). But similarly to the experiments without TAP, the community structure in the sieved material fermenter was clearly different from the one in the HRAS fermenter.

In the experiments without TAP and after establishment of a stable microbial community (after around 20 days), the dominant phyla in the fermenters treating primary sludge or sieved material were *Bacteroidetes*, *Firmicutes* and *Proteobacteria*. Both fermenters harboured 5 abundant families (relative abundance always >3% after day 20), of which they had 4 in common: *Porphyromonadaceae*, *Christensenellaceae*, *Comamonadaceae* and *Rhodocyclaceae* (**SI Table A.1** for the respective relative abundances). The other abundant families found in the primary sludge and sieved material fermenter were the *Ruminococcaceae* and *Propionibacteriaceae*, respectively. The microbial communities in the HRAS fermenter were significantly different, with about twice as many sequences affiliated to the phylum *Proteobacteria* and with a lower relative contribution of the phylum *Bacteroidetes*. *Comamonadaceae*, *Rhodocyclaceae* and *Carnobacteriaceae* composed the dominant families in the HRAS fermenter (**SI Table A.1** for the respective relative abundances). Also, the fermenter treating HRAS was the only one displaying similar community structures between the influent and the reactor.

Application of TAP strongly reduced the relative abundance of the phylum *Proteobacteria* in the fermenters. Higher relative abundancies of *Actinobacteriota* and *Bacteroidetes* were in turn observed in the sieved material and HRAS fermenter, respectively. Concomitantly, the Shannon diversity index decreased from 2.84 to 2.19 for the sieved material fermenter, and from 3.15 to 2.77 for the HRAS fermenter (**SI Table A.2**). The community structure observed in the sieved material fermenter was therefore significantly less complex compared to the one in the HRAS fermenter. The 4 abundant

families identified in the sieved material fermenter accounted on average for 77% of the sequences: *Porphyromonadaceae* (29%), *Propionibacteriaceae* (26%), *Christensenellaceae* (14%) and *Ruminococcaceae* (8%). On the other hand, 6 abundant families were identified in the HRAS fermenter and represented on average 68% of the sequences: *Christensenellaceae* (14%), *Porphyromonadaceae* (14%), *Muribaculaceae* (13%), *Rikinellaceae* (11%), *Ruminococcaceae* (10%), and *Carnobacteriaceae* (6%). In both fermenters, most of the abundant families were dominated by a single genus (**Table 3**). *Paludibacter* and *Christensenellaceae R-7 group* accounted for most of the sequences affiliated to the *Porphyromonadaceae* and *Christensenellaceae*, respectively. In the sieved material fermenter, sequences affiliated to the *Propionibacteriaceae* belonged almost exclusively to the genus *Propionicimonas*. In the HRAS fermenter the *Rikinellaceae* and *Muribaculaceae* consisted mostly of unknown genera, while all of the *Carnobacteriaceae* could be associated to the genus *Trichococcus*. In both fermenters, the *Ruminococcaceae* were the most diverse abundant family. The sieved material fermenter contained similar proportions of *Saccharofermentans* and *Ruminococcus*. In the HRAS fermenter, four genera accounted for 93% of the *Ruminococcaceae*, with *Sporobacter* being the most abundant (**Table 3**).

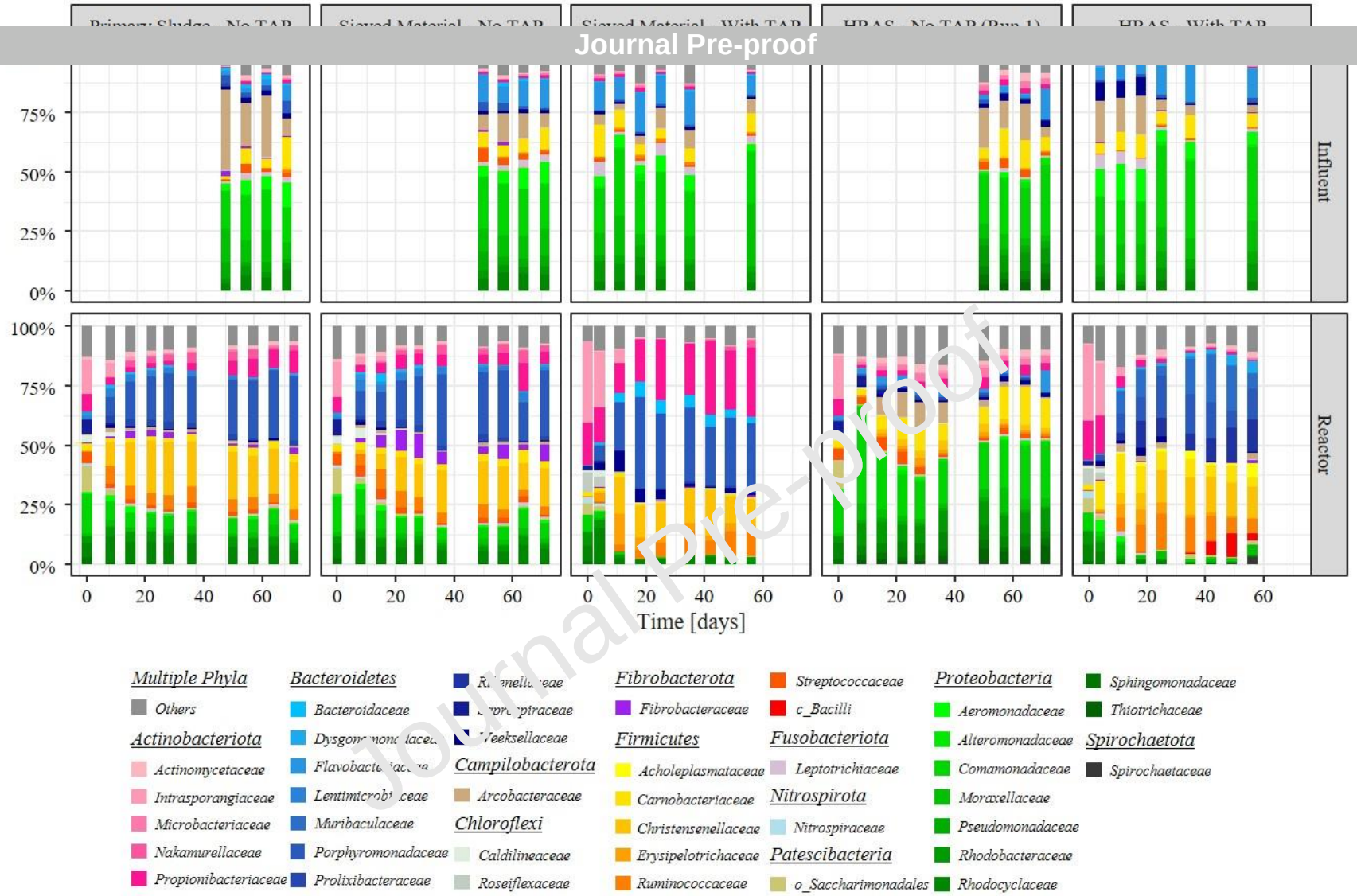

***Figure 3**: Microbial community structures in the influent and reactor samples for all experiments. The plot shows the different taxa at the family level, grouped by phyla. "Others" englobes all families that had a relative abundance < 3 % in all of the samples. No microbial data is available for the 2nd run of the HRAS experiment without TAP.*

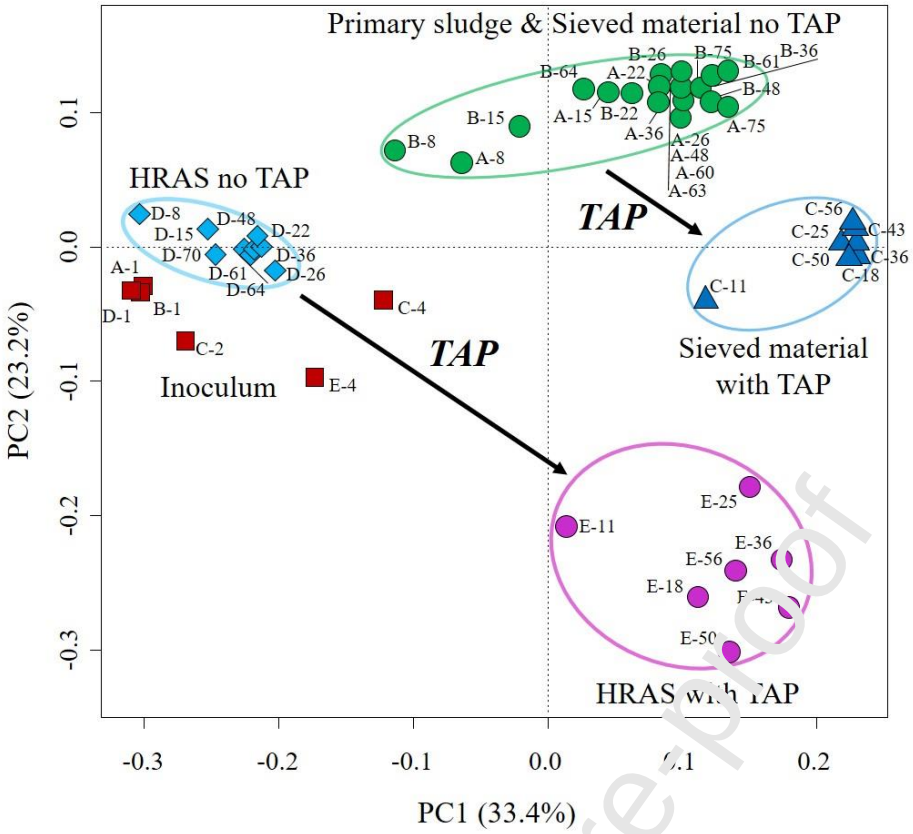

Figure 4: *PCA plot based on Hellinger-transformed relative abundances of the bacterial taxa (family level) present in the different reactors. Samples (dots) appearing close to each other can be expected to be similar in terms of microbial community structure. A: Primary sludge without TAP. B: Sieved material without TAP. C: Sieved material with TAP. D: HRAS without TAP. E: HRAS with TAP. Numbers following the capital letter indicate the day of the experiment. Red squares are the inocula plus samples sharing a high degree of similarity in terms of microbial community structure.*

Table 3: *Relative abundance of individual genera within the abundant families for the fermenters where TAP was applied on influent solids. Only genera whose relative abundance within their family was >5% are shown, the remaining genera were regrouped in "Others". The values in parenthesis show the average percentage of sequences that are affiliated to the different abundant families. Only samples during stable operation (after 20 day) were considered.*

Abundant Families	**Genera**	**Relative abundance within family** Average after day 20 [%]
Sieved Material Fermenter with TAP		
Christensenellaceae (14%)	*Christensenellaceae R-7 group*	100%
Porphyromonadaceae (29%)	*Paludibacter*	100%
Propionibacteriaceae (26%)	*Propionicimonas*	94%
	Others	6%
Ruminococcaceae (8%)	*Ruminococcus*	51%
	Saccharofermentans	42%
	Others	7%
Others (23%)	-	-
HRAS Fermenter with TAP		
Carnobacteriaceae (6%)	*Trichococcus*	100%
Christensenellaceae (14%)	*Christensenellaceae R-7 group*	100%
Muribaculaceae (13%)	*unknown genus*	100%

Porphyromonadaceae (14%)	*Paludibacter*	95%
	Others	5%
Rikenellaceae (11%)	*unknown genus*	98%
	Others	2%
Ruminococcaceae (10%)	*Sporobacter*	54%
	Oscillibacter	12%
	Ruthenibacterium	18%
	Saccharofermentans	10%
	Others	7%
Others (32%)	-	-

3.3 To what extent does the influent solids composition and their pre-treatment impact their solubilisation and the VFA yield?

3.3.1 Solubilisation

The solubilisation of the different types of solids was assessed in a fermenter alone or in combination with TAP (**Figure 5A**). Without TAP, similar solubilisation was observed for the different types of solids: 0.19 ± 0.07, 0.22 ± 0.07 and 0.19 ± 0.06 gsCOD $gpCOD_{in}^{-1}$ for primary sludge, sieved material and HRAS (Run2), respectively. With TAP, the overall solubilisation of HRAS increased significantly, by ~70% (0.32 ± 0.06 gsCOD $gpCOD_{in}^{-1}$), with 99% of the solubilisation then occurring in the TAP unit. In contrast, the overall solubilisation of the sieved material increased by only 25% (0.27 ± 0.08 gsCOD $gpCOD_{in}^{-1}$) while solubilisation occurred still mostly in the fermenter.

3.3.2 Acidification degree and VFA yield

Average acidification degrees and VFA yields were monitored (**Figure 5B and 5C)**. Without TAP, almost full fermentation towards VFAs was observed in all fermenters as indicated by the rather large acidification degrees: > 0.85 for all fermenters. The VFA yields were also similar, with values of 0.16 ± 0.07, 0.19 ± 0.06 and 0.16 ± 0.06 $gCOD_{VFA}$ $gpCOD_{in}^{-1}$ (corresponding to 238 ± 99, 251 ± 74 and 234 ± 93 $mgCOD_{VFA}$ $gVSS_{in}^{-1}$) for primary sludge, sieved material and HRAS, respectively. TAP of the solids significantly reduced the acidification degree of the HRAS (from 0.89 ± 0.05 down to 0.74 ± 0.05) and to a lower extent of the sieved materials (from 0.86 ± 0.04 down to 0.82 ± 0.05). But TAP

still helped to increase the overall VFA yield on HRAS and sieved material by 35% and 20%, respectively (corresponding to 385 ± 75 and 361 ± 124 $mgCOD_{VFA}$ $gVSS_{in}^{-1}$).

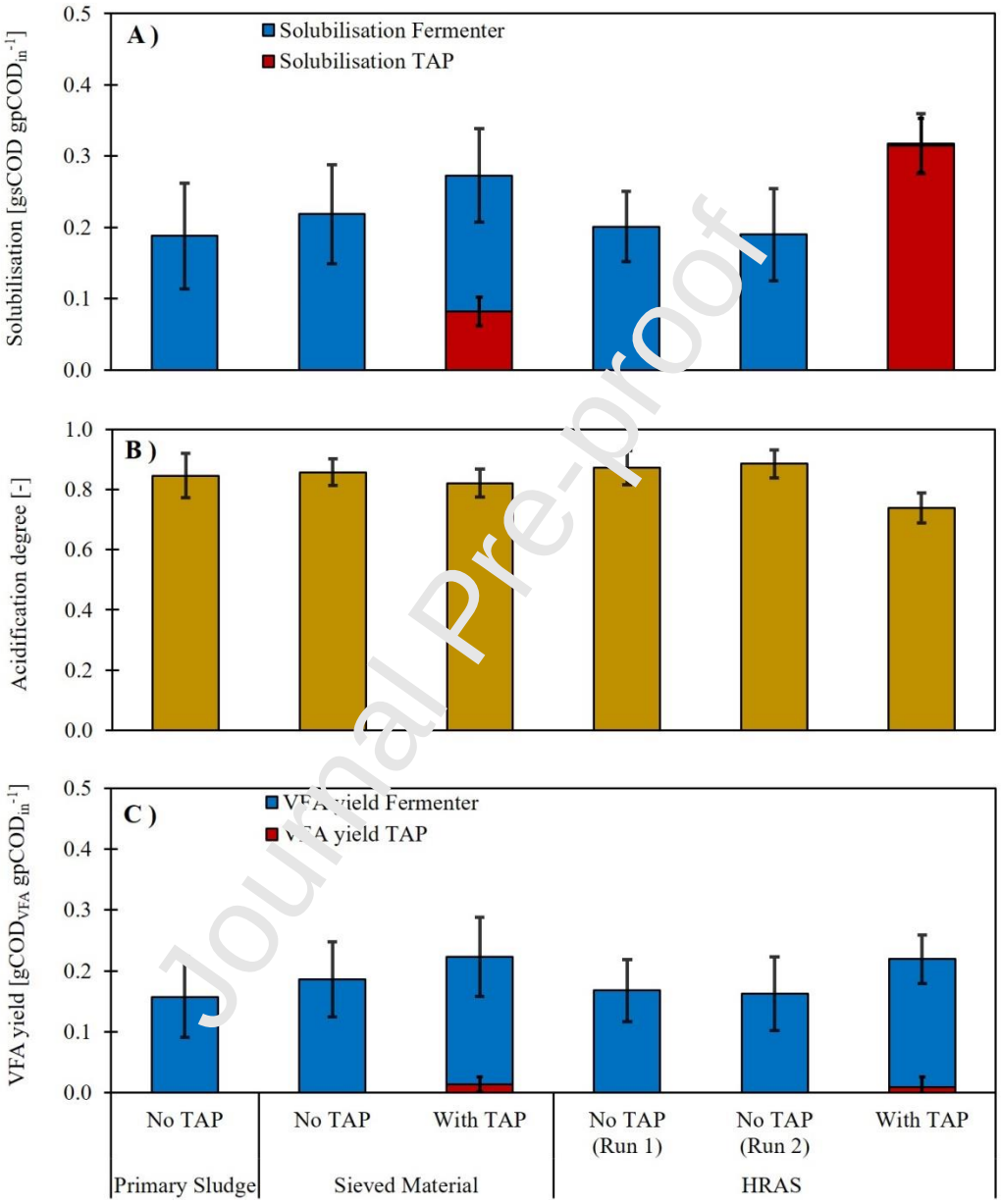

Figure 5: *A) Average solubilisation with the respective contribution of TAP unit and fermenter. B) Average acidification degrees. C) Average VFA yields with the respective contribution of TAP unit and fermenter. Due to operational problems between days 10 – 50 in Run1 of the HRAS experiment without TAP, only measurements between day 60-75 were included in the calculation of average values.*

3.4 How does the composition of solids and their pre-treatment influence the acid composition in the effluent of the fermenters?

The proportions of different VFAs and the nutrient availability were monitored in the effluent of the fermenters (**Figure 6** and **SI Table A.3**). Overall, the effluent VFA composition was governed by the capture technology. Solids captured by the primary settler or the micro-sieve resulted in a VFA composition consisting mainly of acetate and propionate, while HRAS yielded a more complex VFA composition with iso-valerate and iso-butyrate at the expense of propionate. Also, TAP of the solids did not significantly modify the VFA composition. Finally, the nutrient availability in the effluent of the fermenters treating HRAS was much higher than for primary sludge or sieved material.

In the experiments without TAP, acetate was the dominant VFA produced in all fermenters, accounting for >50% of the effluent VFAs during stable operation. For the primary sludge and sieved material fermenters, the remaining VFAs consisted mainly of propionate (~35%), while longer chained acids were negligeable. On the other hand, the systems treating HRAS yielded higher proportions of iso-valerate and iso-butyrate (~20% altogether) while propionate accounted for only ~20% of the effluent VFAs. TAP of sieved material slightly increased the acetate fraction (from 55% up to 63%) at the expense of the propionate fraction (from 36% down to 28%). Contrarily, TAP of HRAS slightly decreased the acetate fraction (from 52% down to 44%) in favour of the propionate fraction (from 22% up to 27%).

Effluent NH_4-N/sCOD and PO_4-P/sCOD of HRAS fermenters (> 100 $mgNH_4$-N $gsCOD^{-1}$ and 25 – 30 $mgPO_4$-P $gsCOD^{-1}$) were 2 respectively 1 orders of magnitude above those of primary sludge or sieved material fermenters (< 1 $mgNH_4$-N $gsCOD^{-1}$ and 2 – 3.5 $mgPO_4$-P $gsCOD^{-1}$).

3.5 Correlations between capture technologies, composition of solids, microbial communities and the resulting VFA compositions

A correlation heatmap was used to evaluate the link between the composition of solids, the microbial community structures and the proportions of individual VFAs in the effluents (**Figure 7**). A redundancy analysis was also performed to support the correlations deduced from the heatmap and to highlight the statistically significant link between the composition of solids and the effluent VFA composition (**SI Figure A.2**).

Compared to primary sludge and sieved material, the HRAS was characterised by a higher nutrient content (nitrogen and phosphorus) and soluble COD fraction (especially when TAP was applied), but lower VSS/TSS. In the heatmap describing the experimental set without TAP, three taxon clusters (A, B and C) were identified (**Figure 7**, left panel). Cluster A contains taxa that correlated positively with VSS/TSS, but negatively with the nutrient contents and soluble COD fraction in the influent. Cluster A therefore includes most of the families that were abundant in the primary sludge and/or sieved material fermenter (*Porphyromonadaceae*, *Christensenellaceae*, *Rhodocyclaceae*, *Ruminococcaceae*, *Propionibacteriaceae*). These families had a strong positive correlation with acetate and propionate, but a negative correlation with iso-acids. In opposition to Cluster A, Cluster C included taxa which correlated positively with the influent nutrient contents and soluble COD fraction, but which correlated negatively with VSS/TSS. Among these taxa were the three abundant families of the HRAS fermenter (*Rhodobacteraceae*, *Carnobacteriaceae* and *Comamonadaceae*). These families correlated positively with iso-acids, and in general, displayed a negative correlation with acetate and propionate. Finally, Cluster B included four taxa that were negatively correlated with VSS/TSS, acetate and valerate.

Four taxon clusters (D, E, F and G) were identified when TAP was applied (**Figure 7**, right panel). Cluster G included taxa showing a strong positive correlation with influent nutrient contents and soluble COD fraction. Among these taxa were abundant families of the HRAS fermenter, such as the *Muribaculaceae*, *Rikenellaceae* and *Carnobacteriaceae*. The same families showed a strong positive correlation with iso-acids, butyrate and valerate. In contrast to Cluster G, Cluster D included taxa that

correlated negatively with influent nutrient contents and soluble COD fraction, such as the *Propionibacteriaceae* or the *Porphyromonadaceae.* While the *Propionibacteriaceae* were abundant in the sieved material fermenter only, the *Porphyromonadaceae* were abundant in the HRAS fermenter as well. Both families correlated positively with acetate and propionate, but negatively with iso-acids, butyrate and valerate. Cluster F included four taxa only, among which the *Ruminococcaceae* and *Christensenellaceae*. Both families were abundant in the sieved material and HRAS fermenter, and positively correlated with propionate. Finally, Cluster E included taxa that were mainly negatively correlated with VSS/TSS, as well as acetate and propionate.

Correlations between the composition of solids, the microbial community structures and the resulting VFA composition were further evaluated by redundancy analysis (**SI Figure A.2**). This analysis suggested that acetate and propionate fractions did not strongly correlate with the composition of influent solids, but that the latter was closely linked to the fraction of iso-acids. The permutation tests yielded p-values of 0.001 which allowed to validate the significance of the models used for the redundancy analyses.

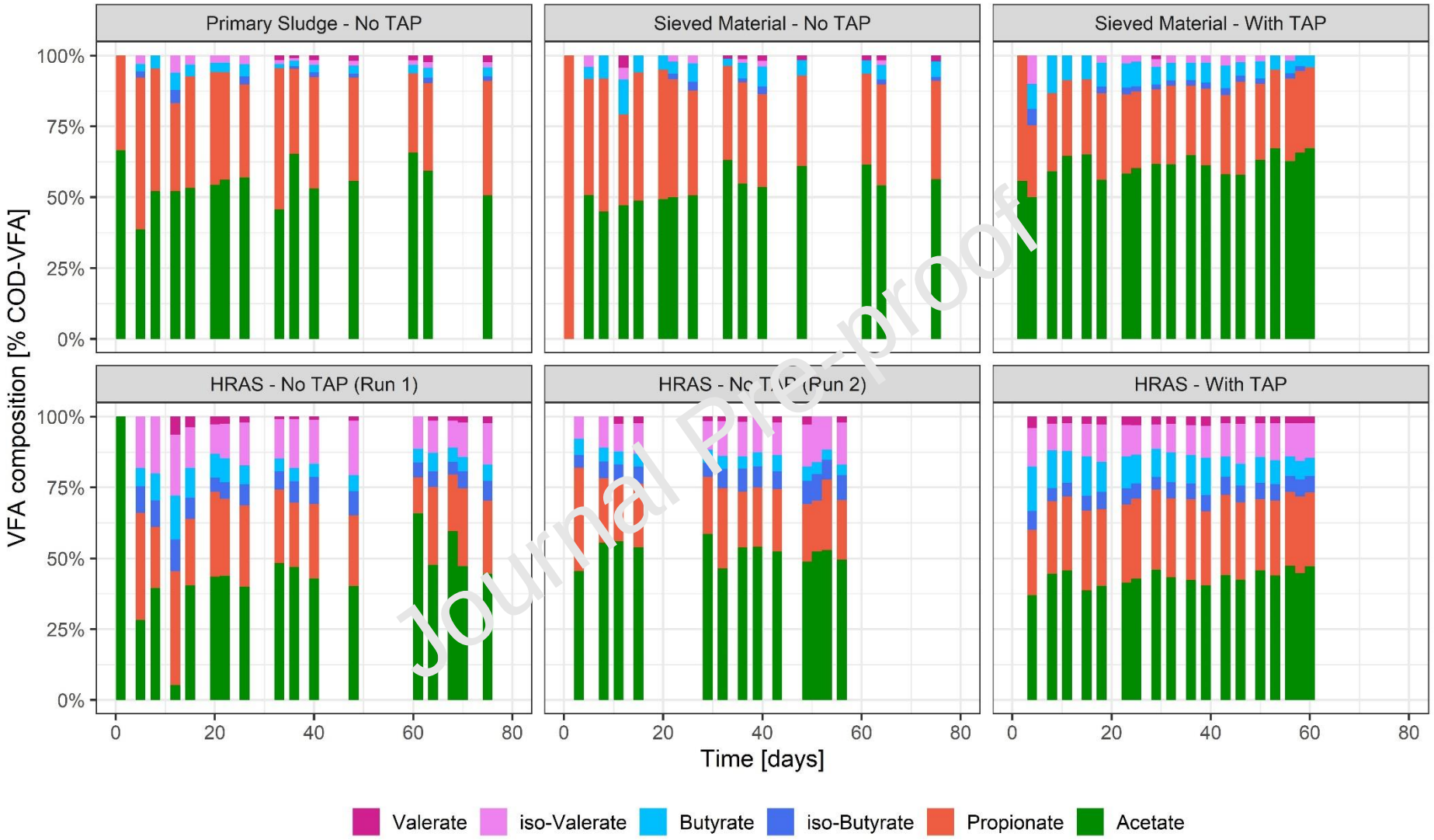

***Figure 6**: Effluent VFA composition (on a COD basis) in terms of acetate, propionate, iso-butyrate, butyrate, iso-valerate and valerate.*

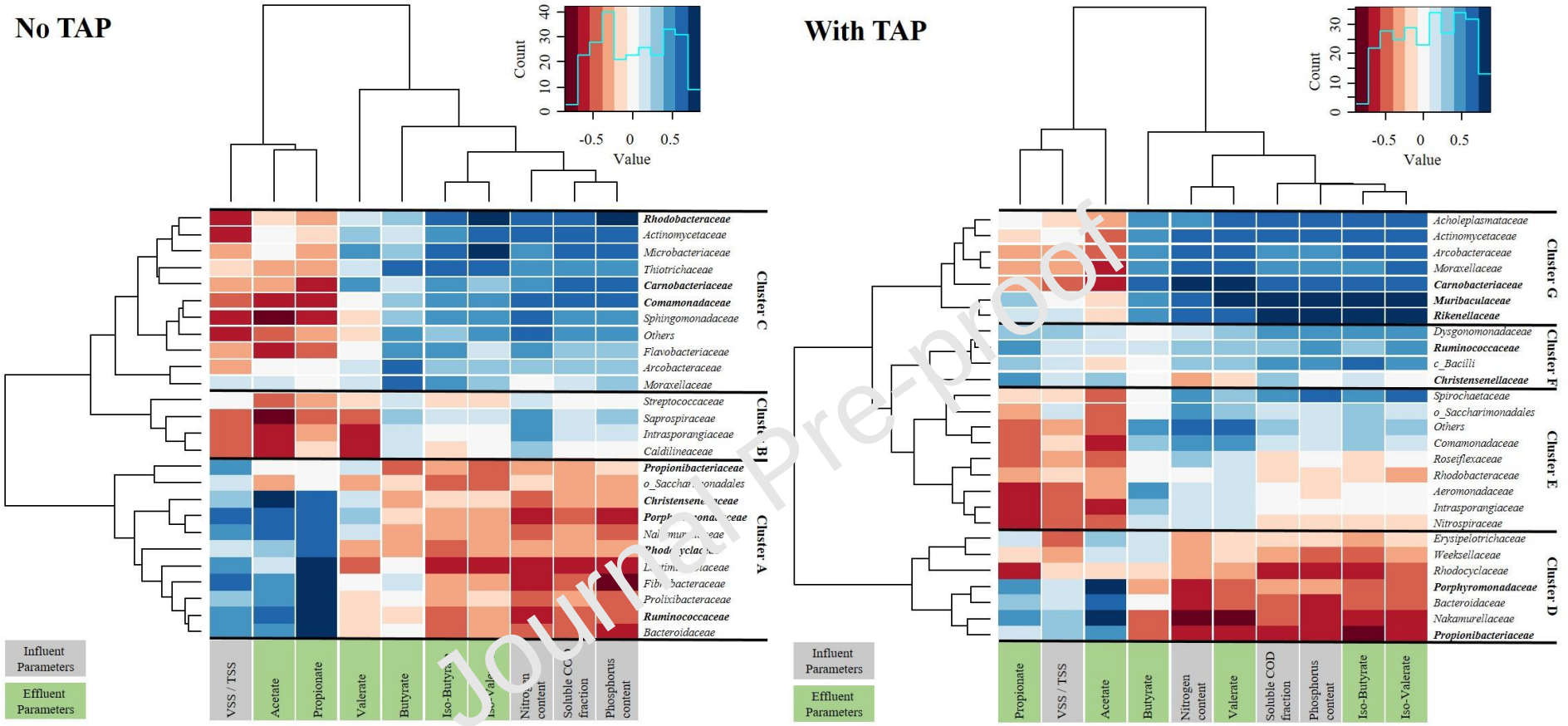

***Figure 7**: Heatmaps showing the correlation between bacterial taxa (in the fermenters) and environmental variables, describing (i) the influent composition (grey), and (ii) the effluent composition (green). Separate heatmaps were generated for the experiments without TAP (No TAP) and with TAP (With TAP). Positive and negative correlation coefficients were colored in blue and red shades, respectively. The dendograms on the left-hand side of the heatmaps cluster the taxa based on their correlation similarity with the different environmental variables. Whenever possible, taxa were specified at the family level. "Others" englobes all families that had a relative abundance inferior to 3% in all samples. Taxa highlighted in bold were abundant (relative abundance always >3% after day 20) in at least one of the fermenters of a specific experiment. The soluble COD fraction was calculated in the influent of the fermenter and therefore, in the effluent of the TAP unit for the experiments with TAP.*

4 Discussion

4.1 The capture technology governs the VFA-profile in the fermenter.

Micro-sieves and HRAS systems might replace the conventional primary settler for the sake of maximising the capture of organic carbon from MWW in the future. To assess how this technological switch might influence organic carbon recovery via the VFA platform, we compared the fermentation of solids from all three technologies under similar operational conditions and using the same MWW source. Such a systematic approach allowed to demonstrate for the first time, that the type of capture technology governs the VFA composition in the fermenters (**Figure 6**). Solids captured with the primary settler or micro-sieve resulted in a similar VFA composition, with acetate and propionate accounting for >90% of VFAs, while fermentation of HRAS entailed increased fractions of iso-acids at the expense of the propionate fraction. The fact that acetate and propionate are major products of fermentation, regardless of the capture technology, is also supported by previous studies investigating the fermentation of primary sludge, sieved material, or HRAS (Cagnetta et al., 2017; Da Ros et al., 2020; Elefsiniotis and Oldham, 1994). Higher amounts of iso-acids however, have only been reported during the fermentation of WAS and HRAS (Cagnetta et al., 2016; Morgan-Sagastume et al., 2011). Our results further demonstrate that both, the composition of the produced solids and the microbial community structures in the fermenters, are very similar for the primary settler and the micro-sieve but very different for the HRAS system (**Table 2, Figure 3** and **Figure 4**). To better understand the relationship between capture technology and VFA composition, it is therefore essential to examine the causal relationship between capture technology, the composition of the resulting solids, and ultimately the community structures within the fermenters.

4.2 Different composition of captured solids explains differences in microbial communities and VFA-profiles in the fermenters.

4.2.1 The mechanism involved in the capture of organic carbon drives the composition of the produced solids.

Technologies based on sedimentation (primary settler) and filtration (micro-sieve) target the capture of settleable and filtrable solids, while HRAS systems rely on microbial growth to capture soluble organics in form of biomass, and also recover solids *via* adsorption onto the flocs (Sancho et al., 2019). Our results indicated that solids captured *via* sedimentation or filtration contained only little biomass and were mainly composed of fibers (**Table 2** and **Figure 2**). Those fibers are typically cellulose from toilet paper, and therefor rich in polysaccharides (Ruiken et al., 2013). In contrast, the HRAS system produced floccular sludge with a high biomass content, as suggested by the high ATP concentrations and visual abundance of nucleic acids (**Table 2** and **Figure 2**). HRAS thus contained larger amounts of proteins and RNA, which can constitute up to 75% of the bacterial cell dry weight (Tchobanoglous et al., 2013). Bacterial cells have typically a nitrogen and phosphorus content of 85 mgN $gpCOD^{-1}$ and 14 mgP $gpCOD^{-1}$, respectively (Tchobanoglous et al., 2013). In comparison, the nutrient contents of HRAS observed in our study (52 – 73 mgN $gpCOD^{-1}$ and 10 – 14 mgP $gpCOD^{-1}$) or in literature (37 – 46 mgN $gpCOD^{-1}$ and 8 – 9 mgP $gpCOD^{-1}$) (Rahman et al., 2019) are lower, which is likely due to the capture of polysaccharide-rich solids (low nutrient content) *via* adsorption. As opposed to HRAS, the primary sludge and sieved material in our study were associated with low nutrients content: 11 – 15 mgN $gpCOD^{-1}$ and 2 – 3 mgP $gpCOD^{-1}$ (**Table 2**). These values are in range with values reported by Ravndal et al. (2018) for raw MWW solids ranging from 100 – 1,000 µm (14 mgN $gCOD^{-1}$ and 2 mgP $gCOD^{-1}$). The same authors also reported that solids > 100 µm (settleable solids) consisted almost exclusively of polysaccharides. Accordingly, low protein (13% of total solids) and lipid (9-13% of total solids) contents are usually reported for primary sludge (Elefsiniotis and Oldham, 1994) and sieved material (Da Ros et al., 2020). Overall, our results demonstrate that (i) primary settlers and micro-sieves produce solids that consist mainly of polysaccharides while (ii) HRAS systems produce solids with high biomass and thus protein content. An important question is

then how the different composition of these solids impacts the microbial community structures within the fermenters.

4.2.2 Composition of the influent solids shapes the microbial community structures within the fermenters

With or without TAP, the structure of the microbial community in the HRAS fermenter was clearly different from the ones in the primary sludge and sieved material fermenters (**Figure 3** and **Figure 4**). Without the TAP, the HRAS fermenter differed from the other two fermenters primarily by having roughly twice the percentage of *Proteobacteria* (~50% vs. ~20%). Previous studies showed that most *Proteobacteria* in anaerobic digesters are residue populations which migrate into the reactor with the influent but remain inactive under anaerobic conditions (Mei et al., 2016; Mei et al., 2017). Since *Proteobacteria* represented ~50% of the microbial community of all our influents, those bacteria were also found in substantial amounts in the fermenters, especially in the one fed with the biomass-rich HRAS (**Figure 3**). Pre-reatments that cause bacterial cell lysis reduce residue populations in anaerobic digesters to negligeable amounts (Mei et al., 2017). Accordingly, application of TAP caused a significant decrease of the *Proteobacteria* fraction in the sieved material and HRAS fermenters, and hence provided an accurate picture of the different anaerobic populations actively involved in the hydrolysis and fermentation of the different solids (**Figure 3**). The solubilisation of the polysaccharide-rich sieved material occurred in the fermenter rather than in the TAP unit, pointing towards microbial hydrolysis (**Figure 5A**). Microorganisms capable of hydrolyzing polysaccharides (saccharolytics) have a clear competitive advantage when these compounds are dominant in solids. Over 40% of the microbial community thus consisted of strictly anaerobic saccharolytic bacteria, such as *Porphyromonadaceae* and *Christensenellaceae* (Krieg, 2015; Morotomi et al., 2012). A further 35% of the microbial community could be assigned to carbohydrate-fermenting bacteria on the basis of the genera represented by *Ruminococcaceae* (*Ruminococcus* and *Saccharofermentans*) and *Propionibacteriaceae* (*Propionicimonas*) (Chen, 2017; Ezaki, 2015; Nielsen et al., 2012). Sieved material thus selected for a rather simple community (4 families accounted for 77% of the sequences) specialised in the degradation of polysaccharides. On the contrary, a more complex community

structure developed in the HRAS fermenter, with 6 families accounting for 68% of the sequences. Since TAP is known to hydrolyze proteins more efficiently than carbohydrates (Rani et al., 2012), HRAS was solubilised to a higher extent than sieved material during pre-treatment (32% *vs.* 8%, respectively) (**Figure 5A**). We can thus assume that the pre-treated HRAS contained larger amounts of hydrolysates (amino acids, simple carbohydrates) which are accessible directly to a wider range of microorganisms compared to proteins or polysaccharides, thus, resulting in a more diverse microbial community structure. 3 of the 6 abundant families in the HRAS fermenter were likely involved in the degradation of proteins or amino-acids: the *Rikinellaceae*, *Ruminococcaceae* and *Muribaculaceae*. Several genera of the *Rikinellaceae* (i.e. *Alistipes*, *Mucinivorans*) are capable of proteolysis or fermentation of glycosylated proteins such as mucin (Könönen et al., 2015; Nelson et al., 2015). Half of the *Ruminococcaceae* consisted of genus *Sporobacter*, which can grow on amino acids and sugars (Nierychlo et al., 2020). Finally, the *Muribaculaceae* family seems capable of using proteolysis products, such as urea, as a nitrogen source. (Lagkouvardos et al., 2019). The dominant saccharolytic populations were notably the same than in the sieved material fermenter (*Porphyromonadaceae* and *Christensenellaceae*) and accounted for ~20% of the sequences. This suggests that the polysaccharide fraction in the HRAS was of similar composition than the sieved material. Overall, our results indicate that (i) a simple community specialised in the degradation of polysaccharides will develop on solids captured by sedimentation (primary settler) or filtration (micro-sieve), while (ii) a more complex community, able to thrive on a wider range of substrates, will develop on biologically captured solids (HRAS).

4.2.3 **Relationship between composition of influent solids, microbial communities and the produced VFAs**

Our results obtained with TAP demonstrate that fermenters fed with sieved material and HRAS shared a common pool of carbohydrate fermenting bacteria, which correlated positively with acetate and/or propionate (*Porphyromonadaceae*, *Ruminococcaceae*, *Christensenellaceae* and *Propionibacteriaceae*) (**Figure 7**). Acetate and/or propionate are known fermentation products of the abundant genera *Paludibacter* (fam. *Porphyromonadaceae*) and *Propionicimonas* (fam. *Propionibacteriaceae*), as well

as of certain members of the *Christensenellaceae* family (Akasaka et al., 2003; Krieg, 2015; Upadhyaya et al., 2016). Contrarily, there is no clear evidence that propionate is a direct fermentation product of any of the abundant *Ruminococcaceae* genera (*Ruminococcus*, *Saccharofermentans*, *Sporobacter*) (Chen, 2017; Ezaki, 2015; Grech-Mora et al., 2015). However, *Ruminococcus* and *Saccharofermentans* are known to produce lactate and succinate, which are the two precursors in the different fermentation pathways for propionate synthesis (Gonzalez-Garcia et al., 2017). Huang et al. (2018) thus hypothesized that an important mechanism in the formation of propionate during fermentation of WAS is the interaction between organisms producing lactate or succinate and organisms fermenting lactate or succinate to propionate. We suggest the same mechanism is responsible for the positive correlation of the *Ruminoccocaceae* with effluent propionate observed in our study. Overall, redundancy analysis of our data showed that acetate and propionate correlated weakly with the influent solids composition (**SI Figure A.2**). Accordingly, important fractions of acetate and propionate have been reported, whether pure cellulose (polysaccharides) or pure gelatin (proteins) were fermented (Duong et al., 2015; Rico et al., 2021). Thus, no significant link can be drawn between influent solids composition and high fractions of acetate and propionate in the effluent.

On the contrary, the formation of iso-acids can be directly linked to the influent solids compositon (**SI Figure A.2**). Iso-valerate and iso-butyrate can be produced by the Ehrlich pathway, with valine, leucin and isoleucin as pre-cursors (Bai et al., 2019). Together, these three amino-acids represent on average 15% of all amino acids found in microorganisms (Moura et al., 2013), which partially explains the higher fractions of iso-acids observed during the fermentation of HRAS in our study (~20% of VFA_{COD}), and WAS in literature (20-30% of VFA_{COD}) (Morgan-Sagastume et al., 2011). Among the abundant families that positively correlated with increasing fractions of iso-acids in the HRAS fermenter equipped with TAP, only the *Rikenellaceae* include genera able to ferment amino acids.

4.3 TAP does not significantly modify the VFA composition but increases solubilisation as well as VFA yields

Our results demonstrate that TAP does not significantly modify the VFA composition while increasing the VFA yield from HRAS and sieved material by 35% and 20%, respectively (**Figure 6** and **Figure 5C**). Similarily, Tan et al. (2012) did not observe a significant variation in the VFA composition while increasing the VFA yield from WAS by ~60% *via* TAP (60°C, pH 11): the only noticeable change consisted in a slight decrease of the acetate fraction (from ~65% down to ~60%) in favour of the butyrate fraction. Also, Pang et al. (2020) found that alkaline pre-treatment (35°, pH 10) of WAS, with and without addition of protease enzymes, did not significantly alter the VFA composition produced in subsequent fermentation batches: the acetate fraction slightly increased (from ~45% to ~55%), at the expense of propionate as well as other acids. Overall, it seems that TAP and other forms of alkaline pre-treatment will result at most in slight variations of the dominant acetate fraction, without notably influencing the overall picture of the VFA-spectrum (**Figure 6**) (Pang et al., 2020; Tan et al., 2012). A possible explanation is that ultimately, similar hydrolysates are converted into VFAs, with or without TAP. The main effect of TAP is actually to initiate the degradation of slowly biodegradable compounds. Polymerised proteins, complex lipids, lignin, and to a minor extent, hemicellulose are solubilised into smaller oligomers (Carrere et al., 2016; Liang et al., 2021). We thus hypothesize that in our experiments, TAP mainly increased the solubilisation kinetics of slowly biodegradable solids into oligomers. However, the break-down of those oligomers into simple monomers was still driven by microbial hydrolysis, ultimately resulting in a similar VFA composition produced from pre-treated solids compared to raw solids.

Application of TAP resulted in a higher overall solubilisation of HRAS (0.32 ± 0.06 gsCOD $gpCOD_{in}^{-1}$) compared to sieved material (0.27 ± 0.08 gsCOD $gpCOD_{in}^{-1}$), confirming that TAP helps solubilising proteins more efficiently than polysaccharides (**Figure 5A)** (Rani et al., 2012). The question is why the significant increase in the solubilisation of HRAS (+70%), went hand in hand with a decrease of the acidification degree (-17%), ultimately limiting the increase of the VFA yield to 35% only (**Figure 5A-C**). A possible explanation is (i) the formation of non-VFA sCOD due to the release

of intermediates otherwise not produced by microbial hydrolysis or (ii) the formation of refractory sCOD resistant to biological degradation. For example, refractory sCOD increased from 4% - 9% during thermal pre-treatment of WAS at 130 – 170° (Toutian et al., 2020). In general, increasing the solubilisation of solids via pre-treatment seems to come at the cost of reduced acidification degrees, ultimately limiting the VFA yield (Bahreini et al., 2020; Morgan-Sagastume et al., 2011). Solubilisation efficiencies up to ~0.4 gsCOD $gpCOD_{in}^{-1}$ were observed when applying high-pressure thermal hydrolysis (6 bars, 160°C) to a mix of primary and activated sludge, or when doping sieved material fermenters with cellulase enzymes. However, acidification degrees ranged between 0.34 – 0.54 and VFA yields barely exceeded 0.2 $gCOD_{VFA}$ $gpCOD_{in}^{-1}$. Therefore, future studies must also focus on characterising the non-VFA fraction of the effluent sCOD in order to better understand the link between the type of pre-treatment, the composition of the fermentation products and the fraction of refractory sCOD produced.

4.4 Practical implications

VFAs can be used directly to enhance biological nutrient removal, or indirectly for the production of valuable end-products such as PHAs, biofuel or microbial proteins (Alloul et al., 2018; Atasoy et al., 2018). Our results indicate that mainly acetate and propionate are produced from solids captured with primary settlers or micro-sieves, while the fermentation of HRAS results in a more complex VFA composition with higher fractions of iso-acids. Acetate and propionate are better electron donors than other VFAs for denitrification (Li et al., 2015). A simple substrate composition is also considered advantageous to produce microbial proteins (Alloul et al., 2018). Primary sludge or sieved material thus represent excellent substrates for improving biological nurtrient removal or for the production of microbial proteins. Also, all of the fermenter's effluents displayed high VFA fractions (0.74 – 0.89 $gCOD_{VFA}$ $gsCOD^{-1}$), making them suitable for PHA-production. The ratio between odd and even-chained VFAs was rather similar for the different solids (0.4 – 0.7 gCOD $gCOD^{-1}$) (**SI Table A.3**), and PHAs produced downstream would therefore have a rather similar composition. Overall, VFA-rich streams originating from the fermentation of primary sludge or sieved material might offer a wider range of upgrade or direct reuse options due to their simple composition.

In WRRFs, a mixture of sieved material and HRAS might be fermented to produce VFAs. An important question is to what extent the fermentation of this blend sludge will lead to a different VFA composition. Ucisik and Henze (2008) found that fermenting a mixture of primary and activated sludge yielded higher butyrate and propionate fractions at the expense of acetate, compared to when both sludges were fermented individually. Mixing different solids is thus not "additive" in terms of produced VFA composition, which implies that additional microbial mechanisms then take place. Consequently, our study does not allow to conclude on how mixing sieved materials and HRAS would modify the VFA composition produced during fermentation.

5 Conclusions

1. The choice of the carbon capture technology drives the VFA composition. Primary settlers, micro-sieves and HRAS systems result in a different composition of the produced solids, which in turn determines the complexity of the microbial community structures in the fermenters and ultimately, the VFA composition.
2. The primary settler and micro-sieve helped capturing polysaccharide-rich solids with low protein content (indicated by their low nutrient content). The resulting microbial community in the fermenter was relatively simple and specialized on the degradation of complex carbohydrates. Ultimately, a simple VFA composition was produced by fermentation of these polysaccharide-rich solids, with acetate and propionate accounting for >90% of the VFAs.
3. HRAS was rich in biomass (and thus proteins), and likely also contained some polysaccharide-rich solids captured via adsorption. The resulting microbial community in the fermenter had a more complex structure and was able to use a wide range of substrates (proteins, amino acids, polysaccharides). Ultimately, the VFA composition was more complex with increased fractions of iso-acids (~20% of the VFAs) equaling the propionate fraction, while acetate remained the main acid (~50% of the VFAs).
4. TAP did not significantly modify the VFA composition and solubilised the biomass-rich HRAS more efficiently (+70%) as opposed to the polysaccharide-rich sieved material (+25%).

But TAP of HRAS also resulted in a significant decrease of the acidifaction degree (from 0.89 to 0.74), ultimately limiting the increase of the VFA yield to 35% only.

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Autorship contribution statement

Antoine Brison: Experimental work, Formal analysis, Investigation, Conceptualization, Data curation, Methodology, Software, Visualization, Writing – original draft. Pierre Rossi: Methodology, Formal analysis, Investigation, Visualization, Software, Writing – review & editing. Arnaud Gelb: Software, Methodology, Investigation, Writing – review & editing. Nicolas Derlon: Conceptualization, Validation, Supervision, Funding acquisition, Resources, Project administration, Writing – review & editing.

Declaration of interests

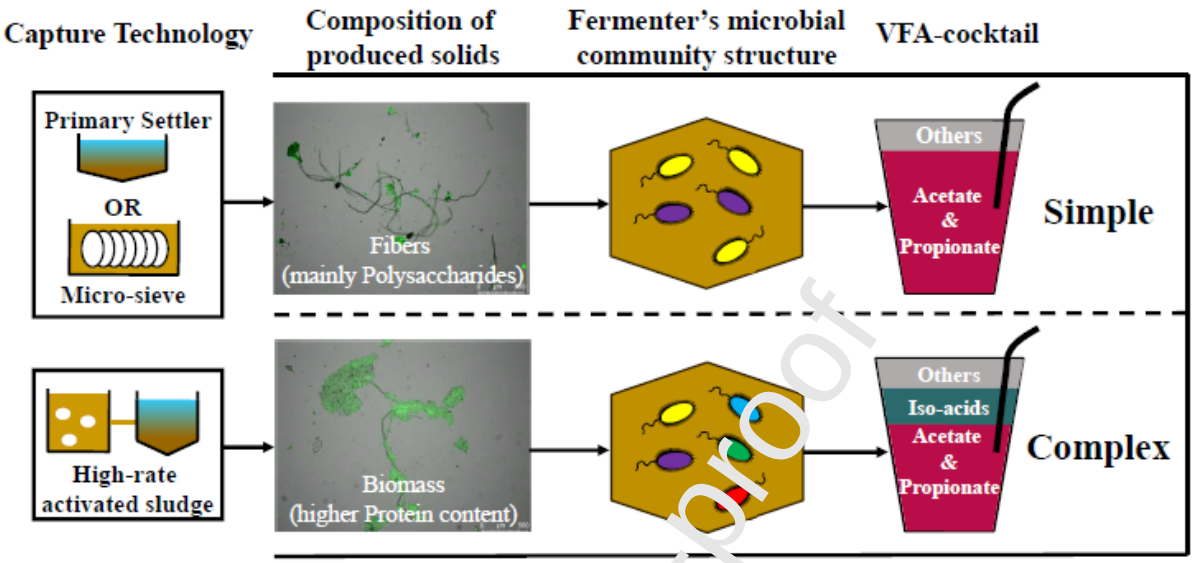
☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:

Sincerely yours,

Nicolas Derlon

Graphical abstract



Highlights

- Effect of carbon capture technology on VFA production/composition was studied
- Settlers and sieves capture polysaccharides, HRAS produces biomass-rich solids
- Fermentation of polysaccharide-rich solids results mainly in acetate and propionate
- HRAS-fermentation yields more complex VFA profile with higher fraction of iso-acids
- Alkaline pre-treatment does not change VFA composition but increases yields