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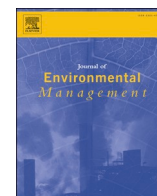
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# The role of growth media on composition, bioconversion and susceptibility for mild thermal pre-treatment of waste activated sludge

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## ABSTRACT

The highly variable characteristics of waste activated sludge (WAS) hinder the comparison of experimental results on WAS bioconversion between the different studies that use excess sludge from different origin. Sludge grown under laboratory conditions with synthetic wastewater as feed showed high resistance to commonly applied pre-treatment techniques, such as thermal pre-treatment. However, a distinctly higher bioconversion of this sludge was recorded compared to WAS from a full-scale wastewater treatment plant (WWTP). The observed results casted concern on the suitability of the experimental laboratory-based data for practice. The physico-chemical and biochemical characteristics of both WAS and lab-grown sludge are dependent on the wastewater characteristics or growth media on which the sludges were grown. The objective of this study was to formulate a growth medium that results into a lab-grown sludge which shows high similarity to the WAS coming from a specific full-scale WWTP in response to a pre-treatment technique. More specifically, in this study we targeted the formation of slowly-biodegradable lab-grown sludge that is similarly responsive to mild thermal pre-treatment with  $H_2O_2$  addition. By comparing real and synthetic wastewaters, we discussed the various wastewater constituents that may lead to a higher degree of recalcitrance of the produced sludge. We then formulated a growth medium, which was fed to a lab-scale activated sludge reactor and evaluated the nutrient removal capacity, as well as the characteristics of the cultivated sludge before and after pre-treatment. Finally, the growth medium was modified to provoke a change in both the bioconversion and in the response to mild thermal pre-treatment. The growth medium proposed in this study resulted in a slowly-biodegradable sludge ( $195 \pm 3.7$  NLCH<sub>4</sub>/kgVS<sub>added</sub>) that after thermal pre-treatment resulted in an increase in methane production of 9 %, which was similar to the WAS coming from the full-scale WWTP. It was concluded that not only the bioconversion but also the response to mild thermal pre-treatment of lab-grown sludge was determined by the composition of the growth media.

## 1. Introduction

Waste activated sludge (WAS) is the most abundant by-product of conventional activated sludge (CAS) wastewater treatment plants (WWTP) for municipal sewage: a biological matrix composed of microbes, metals and countless unknown organic and inert materials (Tchobanoglous et al., 2003). Anaerobic digestion is commonly applied to biodegrade WAS and extensive research has been conducted to maximize its methane production during digestion via pre-treatment methods (Neumann et al., 2016; Pilli et al., 2014).

Nonetheless, the high variability in sewage characteristics and applied CAS systems usually results into unpredictable composition and

characteristics of WAS, casting concern on the reproducibility of lab-scale WAS pre-treatment experiments and limiting the insight on mechanisms underlying the effect of these pre-treatment methods (Gonzalez et al., 2020; Ruffino et al., 2019; Xu et al., 2020). In fact, a systematic comparison between the different WAS pre-treatment techniques demands the availability of sludge with defined non-variable characteristics with a high similarity in physicochemical and biochemical properties with WAS from a full-scale CAS system. For this reason, various researchers cultivated lab-grown sludge that, however, differed substantially in composition and characteristics (Dai et al., 2017b; Xu et al., 2017). In particular, lab-based experimental results have demonstrated that pre-treated sludge, cultivated with synthetic

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wastewater, did not result into an increment in bioconversion similarly as observed for WAS from full scale WWTPs. Since the studied pre-treatment methods generally increase the WAS bioconversion from full scale WWTPs (Gonzalez et al., 2020; Pilli et al., 2014), it was hypothesized that the inherently high bioconversion of the lab-grown sludge severely masked the effectiveness of pre-treatment.

Recent research, clearly showed that the composition of the synthetic wastewater that was used to cultivate the lab-grown sludge, distinctly impacted the characteristics of resulting sludge, although studies addressing the effects on biodegradation are rather scarce (Dai et al., 2017b; Layer et al., 2019; Li et al., 2012; Wang et al., 2018; Xu et al., 2017). Similarly, physicochemical reactions, such as complexation, between some of the components of the wastewater during the formation of sludge, may possibly result in a reduced biodegradation of WAS (Xu et al., 2020). Such reactions might not occur when simple synthetic influents are used to grow the biomass in laboratory experiments.

Considering the above, we consider that, thus far, the used synthetic wastewaters were not suitable for producing lab-grown sludge with a similar recalcitrant organic matrix, leading to non-predictive results of novel WAS pre-treatment techniques. The objective of the present study is to propose and evaluate a new growth medium to produce lab-grown sludge that behaves similarly to WAS from a full-scale CAS system with respect to low-temperature pre-treatment and subsequent anaerobic digestion. In this manuscript, low-temperature pre-treatment refers to the exposure of sludge to 70 °C plus 15 mg H<sub>2</sub>O<sub>2</sub>/g TS.

## 2. Materials and methods

The following research approach was adopted: Firstly, municipal wastewater from 3 CAS systems in The Netherlands were analyzed and their characteristics were used as a baseline for the comparison with several synthetic wastewater recipes that are used in published studies (Aiyuk and Verstraete, 2004; Martín et al., 2010; Ozgun et al., 2013; Xu et al., 2017). Secondly, the rationale behind the composition of the proposed synthetic wastewater (SWW-1) was discussed. Thirdly, the growth medium SWW-1 was fed to a lab-scale activated sludge reactor. The performance of the biological nutrient removal was monitored and the characteristics of the resulting cultivated sludge were compared to those of WAS. (In this manuscript, WAS refers to sludge from the WWTP, while lab-grown is sludge cultivated under controlled conditions.) Finally, a modified version of the proposed synthetic wastewater (SWW-2) was fed to the reactor to induce a change in the response of pre-treatment and provide insight on its mode of action. Details on each step are provided next.

### 2.1. Wastewater and liquors sampling

Samples of settled domestic wastewater and mixed liquors from the aeration tanks were collected from three wastewater treatment plants in The Netherlands: Harnaspolder, in Den Hoorn (GPS 52.013372, 4.317284); Nieuwgraaf, in Arnhem (GPS 51.973766, 6.000757); and Kralingseveer, in Rotterdam (GPS 51.907063, 4.542640). The collection of samples occurred in January and February and Kralingseveer wastewater treatment plant (WWTP) had rainy weather the day before sampling, while the other samples were taken during several days during dry weather condition. The aliquots were then transported to the laboratory within 3 h and stored at 4 °C. All analyses were performed within 36 h of sampling.

### 2.2. Analytics

**COD, solids, VFA, ORP and pH.** Total solids (TS); volatile solids (VS); chemical oxygen demand (COD); and volatile fatty acids (VFA) were measured as described in Gonzalez et al. (2020). pH was measured with a SenTix 940 IDS probe and data was recorded with a multimeter model 3620 IDS (Xylem Inc, U.S.A.). Conductivity was measured with a

TetraCon325 probe and retrieved with a multimeter InoLab® Multi 9620 IDS (Xylem Inc, U.S.A.). Sludge volume index (SVI) was determined using a 1-L Imhoff cone after 30 min of settling based on method 2710D (APHA et al., 2017). Similarly, settleable solids were measured based on method 2540F, while total and volatile suspended solids (TSS and VSS) with method 2540D and E, respectively (APHA et al., 2017). The sample volume for TSS and VSS of wastewater and synthetic wastewater was 40 mL and the samples were filtered through glassfiber filters AP4007000 from (Merck KGaA, Germany) with a nominal pore size of 0.7 µm. A variety of analytical assays were performed using the Hach LCK cuvette test system (Table S1) and were read with a photometer DR 3900 (Hach, U.S.A.).

**Extracellular polymeric substances (EPS) extraction.** EPS extraction was based on the method from Liu et al. (2016). 50 g of sludge diluted at 1:5 with a 0.05% NaCl (w/v) solution at 70 °C were centrifuged at 15000 g for 15 min at 4 °C in a centrifuge model Sorvall ST 16 (Thermo Fisher Scientific, U.S.A.). The supernatant was recovered as the soluble fraction of EPS (S-EPS). The resulting precipitate was resuspended with the saline solution until the total mass of liquor was 50 g. The assay tube was centrifuged again in the same conditions as above and the liquid fraction was retrieved as the lightly bound fraction (LB-EPS). The settled contents in the tube were suspended once more with saline solution at 60 °C. The resulting liquor was mixed with a magnetic stirrer for 30 min at 60 °C. Finally, the heated sample was centrifuged at the mentioned conditions and the supernatant was collected as the tightly bound fraction (TB-EPS). The three EPS fractions were filtered through a polytetrafluoroethylene (PTFE) filter with a pore size of 0.45 µm (Macherey-Nagel GmbH & Co, Düren, Germany). The precipitate obtained after the collection of the TB-EPS, was considered as the pellet fraction.

**EPS composition.** Analyses of protein, humic substances and polysaccharides were performed as shown in Zhen et al. (2019). The standard substrates were bovine serum albumin (BSA); humic acid sodium salt; and D-(+)-glucose, respectively. All the chemical reagents required for determination of EPS were obtained from Sigma (Sigma-Aldrich, U.S.A.), except 98% H<sub>2</sub>SO<sub>4</sub>, which was obtained from Carl Roth (Carl Roth GmbH & Co KG, Germany).

**Inductively Coupled Plasma Optical Emission Spectrometry (ICP-OES).** The preparation of the sample for measuring the concentration of Al; B; Ca; Co; Cr; Cu; Fe; K; Mg; Mn; Mo; Na; Ni; W; and Zn in wastewater, synthetic wastewater and mixed liquor proceeded as follows: 100 mL of sample were filtered through a 200 µm sieve. Approximately 9 mL of the filtered sample were acidified in 4.5 mL of 30% HCl and 1.5 mL of 65% HNO<sub>3</sub> and heated up in a microwave digester model Multiwave PRO (Anton Paar, Austria) for 1 h at 300 °C to solubilize the contents. ICP-OES was performed to the digested sample with a spectrometer model Optima 5300DV (PerkinElmer, U.S.A.) using a sapphire injector and a glass nebulizer. Silicon (Si) was not measured due to problems in acid solubilization. In some samples, Cu and Mn had concentrations with considerable differences compared to the stoichiometric value and interference(s) with other element or compound was suspected (Ranjbar et al., 2012; Shoaee et al., 2012). Thus, for Cu and Mn both the measured and stoichiometric concentrations are presented.

**Specific methane production (SMP) and methane production rate ( $k_{CH_4}$ ).** SMP and  $k_{CH_4}$  were determined as described previously (Gonzalez et al., 2020). Bottles with a working volume of 800 mL were agitated with an oscillatory incubator shaker model Innova 44 (Eppendorf, Germany) at a rotational speed of 120 rpm at 35 °C. The produced biogas was scrubbed with 160 mL of a 3 M NaOH solution and the gas was measured in a gas volume measuring device (BPC, Sweden). Physicochemical characteristics of the sludge samples used for SMP are detailed in Table S2. Thickened WAS was obtained from Kralingseveer WWTP and used as reference. Lab-grown sludge from the sequencing batch reactor (SBR) was collected at several times. The liquor was centrifuged to a concentration of solids similar to the dewatered WAS with a centrifuge model Labofuge 400 (Thermo Fisher Scientific, U.S.A.) at 3500 RCF for 8 min

without the addition of conditioning agents. The inoculum for the SMP tests was obtained from the anaerobic digester of Harnaschpolder WWTP which was operated with a retention time of  $\sim 21$  days at  $37^\circ\text{C}$  and was collected approximately 1 week before the start of each test. Digestate was sieved through a 0.7 mm sieve and pre-incubated between 4 and 7 days at  $35^\circ\text{C}$ .

**Biological oxygen demand (BOD).** BOD was determined with a respirometer Oxitop OC100 controller and Oxitop-C measuring heads (Xylem Inc, U.S.A.) with a detection range of 0–400 mg/L. Around 1 L of sample was saturated with oxygen and diluted to a COD  $\approx 300$  mg/L. 164 mL of the prepared aliquots were added to 3 borosilicate bottles with a working volume of 250 mL. Blank samples in the range of 0–40 mg/L and with a working volume 432 mL were assayed to account for the BOD of the inoculum. Stirring rods were included in each bottle to ensure mixing during the whole assay. Neither nutrient or buffer solutions were added and each bottle was inoculated with 9 mg VSS of activated sludge from the aeration tank of Harnaschpolder WWTP. N-Allylthiourea (Sigma-Aldrich, U.S.A) was used as nitrification inhibitor, thus, the obtained BOD value corresponded to the carbonaceous BOD at the 5th day (CBOD<sub>5</sub>). Finally, 2 pellets of NaOH were added in a separated compartment inside the bottle and then the bottle was sealed with a measuring head. The variation in partial pressure in the bottle caused by the production of CO<sub>2</sub> were detected by the measuring heads and a data point was taken every 20 min and retrieved via ACHAT OC Software (Xylem Inc, U.S.A.). The initial concentration of dissolved oxygen (DO) was around 8 mg/L and the final value was around 4–5 mg/L. The temperature was kept in the range  $20\text{--}21^\circ\text{C}$  along the assay. The COD of the prepared liquors was also measured at the beginning and end of the test to corroborate the data from the respirometer. BOD values of domestic wastewater were obtained from the process operators of the WWTPs.

**Miscellaneous methods.** Particle size distribution (PSD), was measured with a laser diffraction instrument model Microtrac MRB's Bluewave (Microtrac Retsch GmbH, Germany): The sludge was diluted 1:5 with a solution of 0.05% NaCl (w/v) and 2 mL of the resulting solution were analyzed. Turbidity was measured with a Hach 2100N turbidimeter (Hach, U.S.A). Alkalinity was determined via titration with 0.1 M HCl using a volumetric titration system Titrimo SM 702, Metrohm (Metrohm AG, Switzerland): 100 mL of sample were dispensed and mixed with a magnetic stirrer and the amount to acid to reach a pH of 4.5 was recorded and converted into mgCaCO<sub>3</sub>/L equivalents. Micrographs were obtained with a digital microscope model VHX-5000 (Keyence Corp, Japan) with a 100-fold augmentation. Oil and fat method was performed by Silliker Netherlands B.V, (The Netherlands) according to standard NEN 6671. Kjeldahl Nitrogen (Kj-N) was performed by Eurofins Analytico B.V, (The Netherlands) based on method NEN-ISO 5663. Kj-N, TC and TOC for Harnaschpolder and Nieuwgraaf WWTPs and oil and fat from Kralingseveer were not determined due to sanitary restrictions.

### 2.3. Sludge pre-treatment

Pre-treatment of sludge was performed with a heat exchanger composed of an aluminum coil; a water bath with internal recirculation MX20H135-A11B (Polyscience, U.S.A); a peristaltic pump model 520S (Watson-Marlow, U.K); a mixer model 036090000 Series RZR 1 (Heidolph, Germany); and a borosilicate beaker of 600 mL capacity. 450 g of dewatered sludge ( $\sim 8\%$  TS) were pumped from the beaker into the coil which was submerged in the pre-heated water bath at  $71.8^\circ\text{C}$ . Finally, the warmed sludge was returned to the beaker and the cycle was repeated. The temperature of the sludge in the beaker was measured with digital thermometers in two different locations to monitor possible temperature gradients. H<sub>2</sub>O<sub>2</sub> at a concentration of 30% w/w (Merck KGaA, Germany) was dosed to reach a concentration of 15 mg H<sub>2</sub>O<sub>2</sub>/g TS. The addition of H<sub>2</sub>O<sub>2</sub> occurred 10 min after the sludge reached a temperature of  $70^\circ\text{C}$ ; then such temperature was maintained for 30 min.

### 2.4. Synthetic wastewaters

Four synthetic wastewaters (Aiyuk and Verstraete, 2004; Martín et al., 2010; Ozgun et al., 2013; Xu et al., 2017), a synthetic wastewater formulated in our lab (SWW-1), and its modified version (SWW-2) were prepared with lab-grade reagents and tap water. While the first 3 recipes were not originally intended to cultivate sludge, they were claimed to be representative of domestic or municipal wastewater. The four studied recipes and the samples of wastewater from full-scale WWTP and the analysis shown in section 3.2 were used as a baseline for the formulation of the growth-media proposed in this study. Compared to SWW-1, SWW-2 consisted of a) the addition of Sigma-Aldrich humic acid; b) the decrease in concentration of fulvic and humic acids; c) the addition of collagen; d) the decrease in egg-white powder; and e) the removal of bentonite. The resulting COD concentrations were between 300–15,000 mg/L. The formulas were then diluted with tap-water to a COD  $\approx 300$  mg/L and the analytical analyses were performed to the diluted solutions. Finally, to account for the  $\sim 10\%$  variation in COD resulting from dilution error, the values from the analyses were normalized to a hypothetical solution of COD  $\equiv 300$  mg/L. Similarly, the concentration of reagent to achieve a COD  $\equiv 300$  mg/L was calculated and presented in Table S3.

### 2.5. SBR operation

A sequencing batch reactor (SBR) with a working volume of 100 L was operated continuously (Fig. 1). The reactor was inoculated with WAS from Harnaschpolder WWTP to an initial mixed liquor suspended solids (MLSS) concentration of  $\sim 0.5$  g/L. The inoculum was exposed to the proposed growth medium during  $\sim 2.5$  months until an MLSS concentration of  $\sim 4$  g/L. The concentration of ammonium and phosphate in the growth medium started with  $\sim 25\%$  of the dose and increased in proportion to the MLSS. The solids retention time (SRT), organic loading rate (OLR) and food-to-microorganism ratio (F/M) were  $18.6 \pm 2.1$  d;  $0.23 \pm 0.02$  kg O<sub>2</sub>/m<sup>3</sup> d; and  $0.08 \pm 0.01$  kg/kg d, respectively. Such values are typical of lowly-loaded systems with biological nutrient removal (Tchobanoglous et al., 2003). The temperature was kept between  $20$  and  $21^\circ\text{C}$  during the operation and the hydraulic residence time was around 26 h.

The configuration and stage duration of the SBR is shown in Fig. 2. Except for settling and decanting, mixing was provided during all stages with an overhead stirrer model IKA RW16 basic (IKA Werke GmbH & CO. KG, Germany). The SBR performed 3 cycles per day with a duration of 8 h each. Valves, pumps and air compressors were connected to a

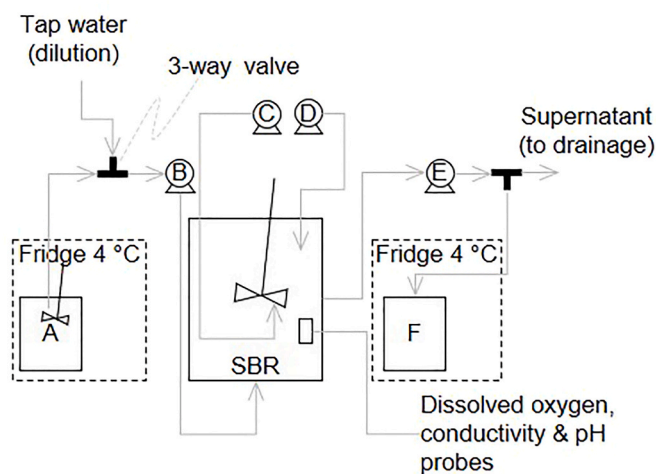


Fig. 1. Experimental set-up. a) concentrated feed tank; b) influent pump; c) air compressor; d) methanol pump; e) effluent pump; f) excess-sludge tank.



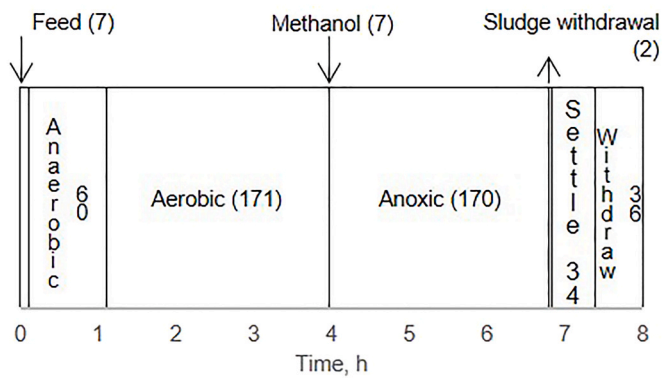


Fig. 2. Sequence of treatment and stage duration. Numbers in parenthesis represent time in minutes.

programmable logic controller LabView software (NI Corp., U.S.A.). pH and conductivity were measured with the probes mentioned above and retrieved with a multimeter model 3620IDS (Xylem Inc, U.S.A.). No pH adjustment was performed during operation.

The synthetic wastewater SWW-1 in Table S3 was concentrated until a COD  $\approx 10$  g/L in a 20 L container and stored at 4 °C for a maximum of 4 days. The variation of COD during storage was  $< 5\%$ , and was considered negligible. An overhead stirrer model 036090000 Series RZR 1 (Heidolph, Germany) was activated during the feeding phase to prevent settling of the concentrated synthetic wastewater during storage. At the start of the anaerobic phase, the concentrated synthetic wastewater was fed from the bottom of the SBR for 7 min and then tap water was added during 60 min to reach a COD  $\approx 500$  mg/L. In the aerobic stage, air was injected with an air compressor via 2 air diffusers located at the bottom of the SBR. Dissolved oxygen (DO) was measured with an optical dissolved oxygen sensor model WQ401 (Xylem Inc, U.S.A.). Air was provided whenever the DO concentration dropped below  $1.3 \pm 0.1$  mg O<sub>2</sub>/L. Due to a delay in the response of the sensor-software interface, the actual DO concentration of the mixed liquor remained between 1 and 3.5 mg O<sub>2</sub>/L along the aerobic stage (171 min). In the anoxic stage (170 min), methanol was added as carbon source for denitrification and at the end of the phase, 1.5 L of excess sludge was withdrawn for 2 min. The liquor was then conveyed to a container and maintained at 4 °C for a maximum of 1 week until sufficient sample was available for the realization of analyses.

## 2.6. Experimental phases

The experiments with the SBR were divided into two phases with the same operational conditions but distinct composition of the used synthetic wastewater. In the first phase, SWW-1 (Table S3) was fed in the SBR for growing the sludge and until day 32 of steady state. From day 32–50 a transition stage was implemented, which consisted in the gradual modification of the feed from the proposed synthetic wastewater to SWW-2. During the second phase (day 50 until the end of the experiment), the SBR was fed with the modified synthetic wastewater.

The rationale behind the second phase was to induce a modification in the biodegradability of the lab-grown sludge by adding recalcitrant organic matter (humic acid and hydrolyzed collagen) in the modified synthetic wastewater. The composition of SWW-2 was in such way adjusted so that its characteristics remained similar to SWW-1. For instance, a portion of the technical-grade mixture of fulvic (17 %) and humic (4 %) acids in SWW-1 was replaced by Sigma-Aldrich humic acid in SWW-2. Similarly, about one-fourth of the egg-white in SWW-1 was replaced by hydrolyzed collagen in SWW-2 and bentonite and iron were removed from the wastewater because the analytical-grade humic acid already contained those metals.

Along the SBR operation, pH; ORP; and conductivity were monitored

on-line. COD, SVI, solids, ammonium and orthophosphate were determined twice a week to assess the performance of nutrient removal. Finally, samples of the cultivated sludge corresponding to the 1st; 2nd; 4th; 4.5th; and 5th; SRT of operation were collected for the realization of TS, COD, EPS, VFA, SMP and ICP-OES analyses as described above.

The software used to generate the graphs were MS Office (Microsoft Corp., U.S.A) and Adobe Photoshop (Adobe Inc., U.S.A.). Data of this study is available at [dataset] Gonzalez (2021).

## 3. Results and discussion

### 3.1. Characteristics of domestic wastewater

It is known that domestic wastewater has unique characteristics depending on location, living habits and seasonal patterns, among others. For instance, the wastewater from Kralingsveer WWTP had a 50 % higher concentration of total solids and inert suspended solids compared to the other plants (Table S4). Similarly, a 3-fold higher concentration of phosphates, Al, Fe, Mg and a 2-fold higher concentration of Ca were detected. Such differences illustrate the unique and variant composition of wastewater, even for locations  $< 15$  km apart. However, there were also similarities in composition; the organic fraction of all 3 wastewater samples consisted largely of proteins, humic substances, carbohydrates, and lipids. Reports from Norwegian, Chinese and American domestic wastewater samples coincide, although data about the concentration of humic substances and metals is rather scarce (Huang et al., 2010; Tchobanoglous et al., 2003). Despite its uniqueness per location, domestic wastewater is expected to be composed of a mixture of compounds that is related to basic human activities such as sanitation and food preparation.

### 3.2. Comparison between domestic and synthetic wastewater

Residuals generated by basic human activities comprise fibers, urea, humic-like substances and metals. However, the recipes of synthetic wastewaters lack or have considerably lower concentrations of humic substances and metals (Table S4), which likely has a strong influence on the characteristics of lab-grown sludge (Aiyuk and Verstraete, 2004; Layer et al., 2019; Xu et al., 2017). In the framework of our present research, we propose to provide a recipe of a new synthetic wastewater that includes constituents which may result into the cultivation of a slowly-biodegradable sludge as discussed below.

#### 3.2.1. Substrate biodegradation

The lower oxygen consumption rate in the carbonaceous BOD (CBOD<sub>5</sub>) assays of the growth media from Martín et al. (2010); Aiyuk and Verstraete (2004) and SWW-1, suggests a slower biodegradation of the substrate compared to the other two synthetic wastewaters (Fig. 3). Moreover, the former recipes had a CBOD<sub>5</sub>/COD ratio more similar to the sampled domestic wastewater (0.37–0.52 vs 0.4–0.6, respectively).

Table S3 shows that starch is the dominant carbon source in the studied synthetic wastewaters. However, starch in domestic wastewater is rapidly hydrolyzed and fermented to VFAs in the sewers, particularly at elevated temperatures, before arriving to the WWTP (Henze et al., 1997). Instead, fibers such as cellulose, hemicellulose, and lignin accounted for  $\sim 20\%$  of TOC in a Chinese domestic wastewater (Huang et al. (2010)). Such polymeric substrates are more refractory to biodegradation (Wang et al., 2018), which delays their utilization. In addition, in order to hydrolyze these more complex polymeric substrates, different kinds of (exo-)enzymes are expected to be produced by the micro-organisms, which may result in a higher production of extracellular polymeric substances (EPS) in activated sludge compared to systems which are fed with more easily degradable substrates (Cadoret et al., 2002; Li and Yang, 2007; Wang et al., 2018). The concentration and composition of EPS is particularly relevant to the biodegradation of WAS because of its high concentration in the sludge, its low

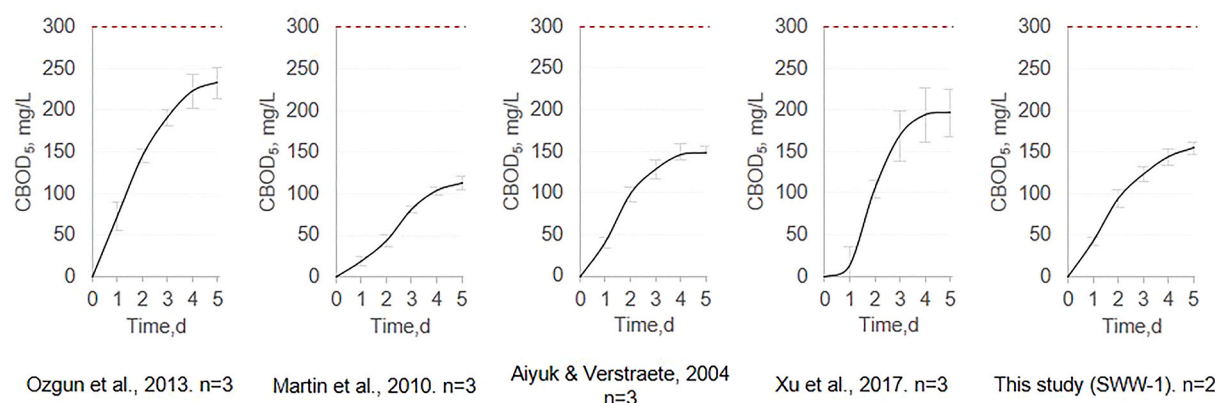


Fig. 3. CBOD<sub>5</sub> of synthetic wastewater samples. Dashed line is the maximum theoretical value.

biodegradation, and its role in agglomerating the cellular biomass (Guo et al., 2020; Li and Yang, 2007).

### 3.2.2. Complexation reactions

**3.2.2.1. Aromatic compounds.** Lignocellulosic matter is present in domestic wastewater in the form of residues of food waste and toilet paper in large parts of the world. The bond that lignin forms between labile organics, results into slowly-biodegradable moieties: while cellulose and hemicellulose are biodegradable, their encrustation with lignin results into recalcitrant lignocellulosic compounds (Hu et al., 2016). Similarly, the formation of hydrogen bonds between the carboxyl group of proteins and the hydroxyl groups of tannins and lignin also results into recalcitrant molecules (Zahedifar et al., 2002).

As already mentioned, the synthetic wastewaters studied in this manuscript were deficient in humic substances (Fig. S1). Humic and humic-like substances in domestic wastewater originate from the reaction between carbohydrates and proteins during food processing and from industrial operations such as leather and wood processing (Kliaugaite et al., 2013; Peschel and Wildt, 1988) and from vegetable waste. Humic-substances are not only recalcitrant –accounting for 3%–28 % of the dissolved organic matter in effluents of WWTP (Kliaugaite et al., 2013; Lipczynska-kochany, 2018), but they are also reactive as chelating agents, electron donors and acceptors and as enzyme immobilization agents, among others (Li and Yang, 2007; Wang et al., 2018). Actually, EPS in sludge comprises of a substantial concentration of humic substances and their absence in the growth medium has been related to a deficient formation of a humic fraction in lab-grown sludge (Labelle et al., 2011; Xu et al., 2017).

**3.2.2.2. Metals.** The concentration of metal-ions adsorbing onto the sludge matrix depends on the influent concentration and the metal dosage in the WWTP, e.g., phosphate removal or flocculation (lime, iron or aluminum). Drinking water can have different concentrations of calcium and magnesium (hardness). The type of sewerage system, i.e., combined or separated, will largely determine the concentration of trace metals such as copper, lead, iron, zinc, and manganese. In addition, domestic and industrial activities contribute to the metal loading that ultimately arrives to the sewers.

The bivalent cation bridging (BCD) model explains the electrostatic attraction between the negatively charged functional groups in the EPS of sludge and multivalent cations (Higgins and Novak, 1997a; Neyens and Baeyens, 2003). Li et al. (2012) observed that certain cations were selectively located in specific fractions of the EPS of sludge. As the pellet fraction of WAS is assumed to be especially recalcitrant, the presence of specific bridging cations in the pellet could be pivotal for the formation of a slowly-biodegradable sludge. Examination of sludge from Kralingseveer WWTP showed that >99 % of Al and Fe in sludge was located

in the pellet fraction (absolute concentrations are shown in Fig. S2). In contrast, only 81 % and 59 % of the total pool of Ca and Mg were detected in the pellet, suggesting a possible substantial role of Al and Fe in the assembly of this fraction. (In some WWTPs, both Al and Fe are added as coagulants to bind organics.) The estimation of the charge equivalents also suggests a substantial contribution of Al and Fe in the assembly of the recalcitrant pellet fraction of WAS (Table 1).

The role of cations in the activated sludge process is not constrained to a mere bridging effect: Both K and Mg are required as counter ions for biological phosphorus removal (Bracklow et al., 2007; Rickard and McClintock, 1992). Moreover, metal-organic complexes possess a higher bond energy compared to the non-complexed components (Guo et al., 2016; Xu et al., 2020). For instance, the non-covalent bond (electrostatic interaction and hydrogen bonding) between bovine serum albumin (BSA) and silica (SiO<sub>2</sub>) resulted into a high structural stability of BSA and ultimately diminished the production of biogas during anaerobic digestion (Dai et al., 2017a).

### 3.2.3. Trace elements

Some trace elements are essential to mediate biochemical reactions but their excessive concentration may result into inhibition of biological processes (Hendriks et al., 2018; Lipczynska-kochany and Kochany, 2009; Xu et al., 2020). Whether the sewer system is combined or separated might have a prominent effect in the concentration of trace elements. Most of the studied synthetic wastewaters had a deficiency of trace elements compared to the samples of wastewater in this study, the latter being originated from combined sewer systems (Fig. S3). On the other hand, the formulas of Martín et al. (2010) and Ozgun et al. (2013), had a considerably higher concentration of cobalt compared to domestic wastewater. Based on the concentrations and composition of trace elements in wastewater and on the growth requirement for anaerobic organisms (Hendriks et al., 2018), a new trace element solution was formulated (Table S5) and included in the proposed synthetic wastewater.

**Table 1**  
Charge equivalents in the pellet fraction of WAS.

Element	Assumed valence	μEquiv/gTS
Ca	2	1695
Mg	2	1067
Fe	2	1623
Al	3	1329

### 3.3. Impact of growth media on methane production of pre-treated lab-grown sludge

#### 3.3.1. Steady-state and macroscopic characteristics of lab-grown sludge

The SBR was inoculated with fresh aerobic sludge from a CAS system and exposed to the proposed growth medium until ~4000 mg MLSS/L was reached, which took ~2.5 months (time 0 in Fig. 4). The SBR was operated for one solids retention time (SRT = 21 days) to renew the biomass of the reactor before the start of the biodegradation (SMP) assays. Such start-up strategy ensures that the cultivated sludge responds to the characteristics of the growth medium. The 'feed transition' time in Fig. 4 (from days 32–50), represents the progressive shift from SWW-1 to SWW-2 in Table S3, which was fed until the end of operation.

The SVI mostly remained above 100 mL/g, although it increased temporally until 175 mL/g apparently as a response to the modified medium (Fig. 4). Such high SVI could be explained by the polymeric nature of the growth medium used (Adler and Holliger, 2020; Martins et al., 2011). A precipitate composed of black solids (similar in appearance to the humic acid used in SWW-2) was evident around day 70 (Fig. S4).

#### 3.3.2. Extracellular polymeric substances (EPS) and volatile fatty acids (VFA)

It was impossible to assess any possible change caused by the growth medium in the EPS of sludge, as EPS measurement was only performed during the second phase of the experiment (at 4th and 4.5th SRT). Both the sludge grown with SWW-2 and WAS had a similar spatial configuration of EPS for both non-treated and pre-treated samples (Fig. S5). Moreover, similar concentrations and speciation of VFAs were detected during the entire experimental period, with no obvious effects caused by the modified medium (Fig. S6).

Overall, the results on EPS and VFA suggest that the lab-grown sludge had a similar composition and structure compared to WAS. The presence of a humic fraction in all the EPS layers of the lab-grown sludge is considered relevant (Fig. S5b), as such fraction was absent in a previous experiment (Labelle et al., 2011). Also, there was a similar concentration and speciation of VFAs during the SMP tests of the pre-treated lab-grown sample compared to WAS (Fig. S6a' and b').

#### 3.3.3. Trace elements and metals

While the concentration of trace elements was similar to WAS (Fig. S7), Ca and Na were almost doubled in the lab-grown sludge (Fig. S8). Since several reagents in the synthetic wastewater were sodium-based, calcium chloride was included to circumvent the pervasive effect of Na in the bridging of sludge (Higgins and Novak, 1997b). However, the addition of humic acid sodium-salt in the modified

medium ultimately resulted into a progressive increase in sodium and a decrease in calcium in the cultivated sludge (Fig. 5). As a consequence, the monovalent-to-divalent cations ratio (M/D) –which is based on the charge equivalents of Na; K; Ca; and Mg of the cultivated sludge, increased (Fig. 5).

#### 3.3.4. Methane production rate ( $k_{CH4}$ )

Substantial discrepancies were observed on the methane production rate. Fig. 6a and b shows that the rate of methane production from the rapidly-biodegradable fraction of sludge ( $k_{CH4-Rapid}$ ), remained similar or even decreased after the exposure to pre-treatment. This result is in clear conflict with the observations from WAS, as increments are commonly reported (Fig. S9) (Gonzalez et al., 2020; Pilli et al., 2014).

The findings about methane production rate of the slowly-biodegradable fraction of lab-grown sludge ( $k_{CH4-Slow}$ ) can be divided in two parts. Firstly, the  $k_{CH4-Slow}$  of the non-treated sludge tended to increase at the end of the operation, which coincides with the application of the modified medium (Fig. 6c). This suggests that the sludge cultivated with SWW-2 was more labile and easier to hydrolyze than the sludge grown with SWW-1. Secondly, the application of pre-treatment increased the  $k_{CH4-Slow}$  of sludge except for the last sample, in which even a notorious decrease was detected (Fig. 6d).

As the application of low-temperature pre-treatment in WAS often derives into increments of methane production rate of both the rapidly and slowly biodegradable fractions (Fig. S9) (Gonzalez et al., 2020; Pilli et al., 2014), it is clear that the lab-grown sludge had a substantially distinct response to pre-treatment compared to the WAS. This deviation poses a limitation on the utilization of the cultivated lab-grown sludge for studies on the kinetics of biogas production. In addition, it was clear that neither the modification of the spatial distribution of EPS or the concentration of VFA were satisfactory indicators for methane production rate.

#### 3.3.5. Specific methane production (SMP)

Lastly, the specific methane production (SMP) of lab-grown sludge was clearly influenced by the characteristics of the medium (Fig. 7). The sludge cultivated with SWW-1 (1st and 2nd SRT) demonstrated an increased specific methane production as a result of pre-treatment. This response was similar to the response obtained in WAS under similar pre-treatment conditions (Gonzalez et al., 2020; Pilli et al., 2014). On the other hand, the sludge grown using SWW-2 resulted into a null response to pre-treatment. Fig. 7 shows that while the SMP of the pre-treated samples from 4th to 5th SRT remain similar, the non-treated sludge became more biodegradable. Ultimately, the SMP of both the non-treated and pre-treated samples became similar and pre-treatment apparently became ineffective.

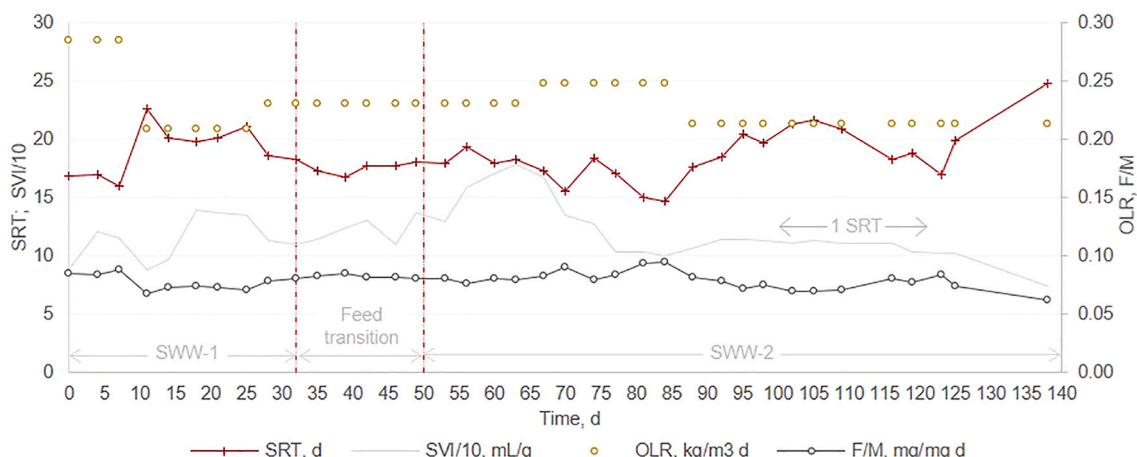


Fig. 4. Operational parameters and phases during the experiment.



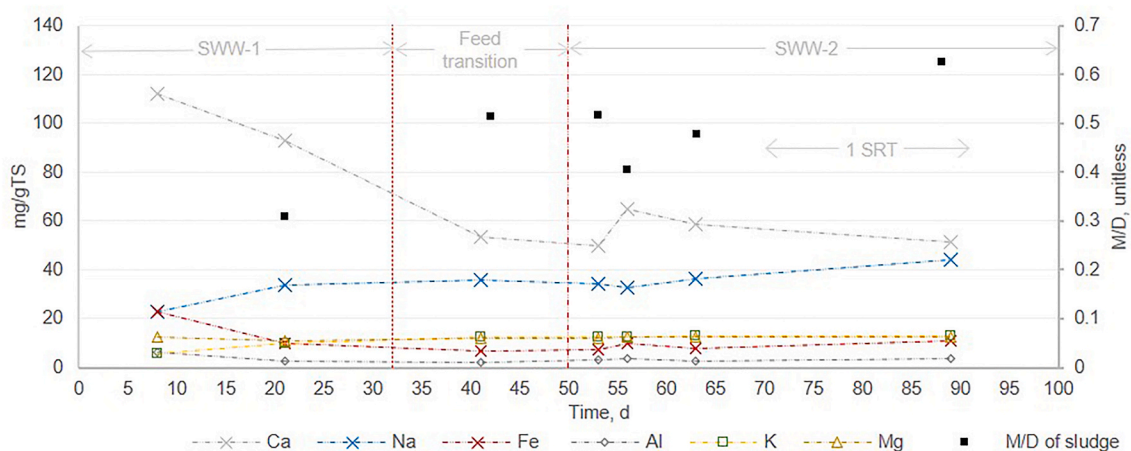


Fig. 5. Concentration of some metals and M/D ratio of the lab-grown sludge during operation.

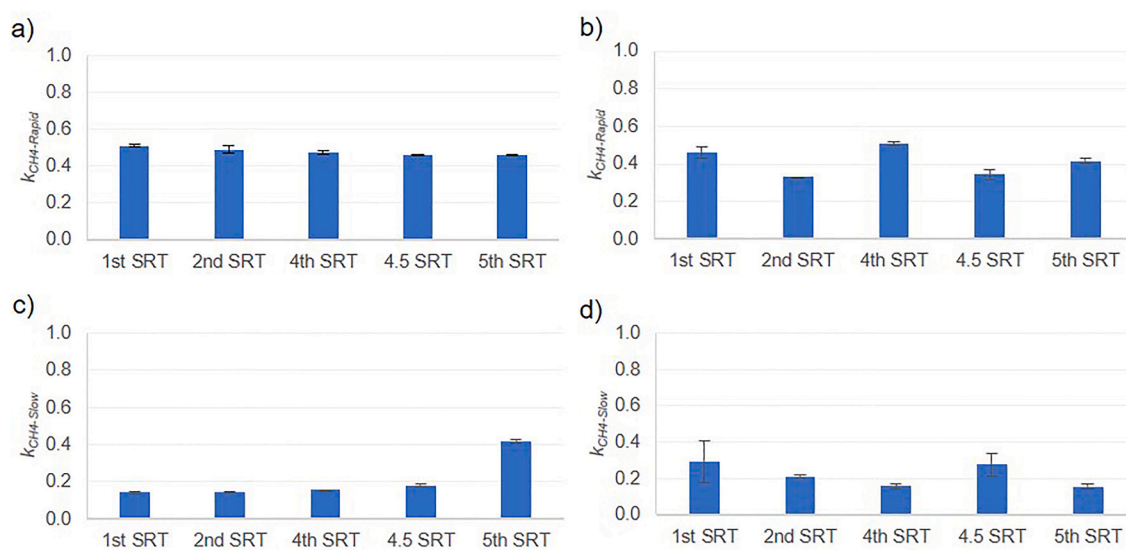


Fig. 6. Methane production rates, a) Non-treated, rapid; b) pre-treated, rapid; c) non-treated, slow; and d) pre-treated, slow. Units in  $d^{-1}$ .

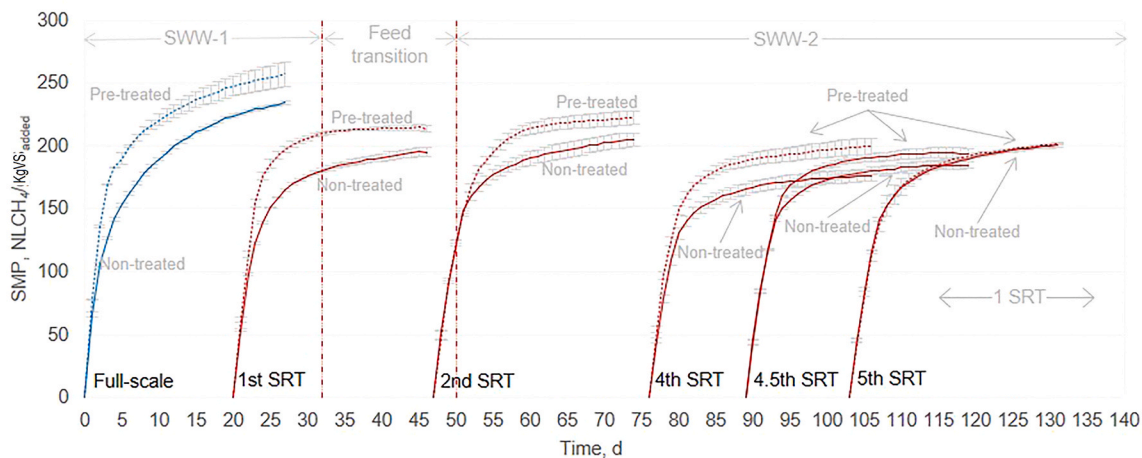


Fig. 7. SMP of full-scale and lab-grown sludge.



### 3.4. Overall discussion

#### 3.4.1. Requirements for synthetic municipal wastewater

The incorporation of polymeric substrates such as cellulose and albumin in the synthetic wastewater was expected to result into a higher concentration of EPS in the cultivated sludge. Likewise, complex aromatic compounds such as lignin and humic substances together with a variety of metals might have resulted in increasing chemical bridging and complexation reactions. The addition of more complex organic compounds together with a sufficient level of trace metals and a diverse and active inoculum, were expected to result in slowly-biodegradable sludge.

#### 3.4.2. Effect of growth-media on methane production of (pre-treated) lab-grown sludge

The evidence presented in this study suggests that not only the biodegradation of lab-grown sludge varied according to the characteristics of the growth media, but also that the effect of pre-treatment depended on the inherent biodegradation of the slowly-biodegradable fraction of the sludge (Fig. 7). As several components from the growth medium were modified during the second phase of the experiment, it is impossible to allocate the observed effects on SMP to a specific constituent of the synthetic wastewater. However, two possible mechanisms might explain why the pre-treatment was unable to increase the SMP of the lab-grown sludge during the end of the operation:

**3.4.2.1. Increase in M/D ratio.** During feeding of SWW-2, the concentration of sodium embedded in the sludge increased, while the concentration of calcium decreased. Overall, such modifications resulted into a progressive increase in the M/D ratio (Fig. 5). A previous report found the M/D ratio to be relevant for flocculation and settling properties (Higgins and Novak, 1997c). The observation was mechanistically explained by an ionic-exchange mechanism in which sodium displaced calcium in the sludge matrix, which affected the concentration of bound protein in sludge (Higgins and Novak, 1997a). This was supported by the observation that an increased sodium concentration was related to a decrease in the bound protein concentration. On the other hand, the increase in Ca and Mg in the feed was associated with an increase in the bound exocellular protein concentration (Higgins and Novak, 1997a). In the present study, a decrease in bound protein might explain the loss of performance of pre-treatment, although this is speculative since EPS measurements were carried out only during the second phase of the experiment (addition SWW-2).

**3.4.2.2. Possible enzyme-humic substances complexation.** Another possible mechanism is the modification of the ratio of fulvic and humic acids. The SWW-1 included a mixture of 17 % fulvic and 4 % of humic acid, while SWW-2 contained a substantial concentration of humic acid sodium-salt (Table S3). We speculate that the biological availability of metals was decreased by the higher concentration of humic acid that may have acted as a chelating agent (Lipczynska-kochany and Kochany, 2008). The loss in performance in P-removal observed during the second phase (Appendix and Fig. S10), could be provoked by a lower availability of K and Mg (Rickard and McClintock, 1992). In addition, a higher chelation could have diminished the bridging effects in sludge, resulting into a weaker assembly that possibly induced the higher biodegradation of the cultivated sludge. Such effect has been observed after the addition of the chelator EDTA to WAS, in which the sequestering of iron played a key role in the increase of biodegradability of WAS (Xu et al., 2017). Thus, the blackish solids observed during the second experimental phase might be the result of precipitated humic acid-iron complexes (Fig. S4) (Boguta et al., 2019).

Since the above possible mechanisms involve metals, it is probable that metals are a pivotal factor predicting not only the biodegradation of sludge (Xu et al., 2017), but also in the effectiveness of the pre-treatment

at the conditions implemented in this study.

### 4. Conclusion

The evidence presented in this study suggests not only that the biodegradation extent of lab-grown sludge was determined by the composition of growth medium, but also the extent of the effectiveness of the applied pre-treatment. The lab-grown sludge had a spatial configuration of EPS, pattern of acidification and specific methane production that was comparable to WAS before and after pre-treatment. However, substantial differences were observed for the rate of methane production, indicating a dissimilar kinetic response compared to WAS. The cultivation of a 'constant-quality' and a slowly-biodegradable sludge that responds to pre-treatment is regarded as a prerequisite for systematic comparison of pre-treatment methods and their impact.

### Author contributions

Adrian Gonzalez: Conceptualization; formal analysis; methodology; investigation; validation; writing – original draft. Jules B. van Lier and Merle de Kreuk: Funding acquisition; project administration, resources, supervision; writing – review & editing. All authors have approved the final version of the manuscript.

### Declaration of competing interest

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

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### Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.jenvman.2021.113491>.

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