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TITLE:

Microbial-chemical indicator for anaerobic digester performance assessment in full-scale wastewater treatment plants for biogas production

SHORT RUNNING TITLE: Methanogens and biogas yield

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GRAPHICAL ABSTRACT

AD is critical to WWTP environmental sustainability and can be an energetically self-contained treatment process. However, AD has only been partially researched due to the lack of knowledge about its potential and the suboptimal valorisation of biogas produced with traditional co-generation systems. This work is focused on developing an AD management tool that includes microbial indicators assessed by biomolecular methods. Finally, a performance index strictly correlated to biogas production is proposed.

ABBREVIATIONS:

AD: anaerobic digestion

MN: total methanogens

SRB: sulphate-reducing bacteria

TotBact: total bacteria

WWTP: wastewater treatment plant

VSS: volatile suspended solids

TSS: total suspended solids

COD: chemical oxygen demand

OC: organic carbon

TC: total carbon

PI: performance index

ABSTRACT

Anaerobic digestion was introduced into wastewater treatment plants several years ago, but anaerobic digestion performance has not yet been achieved. The variability of the microbial community in digesters is poorly understood, and despite the crucial role of anaerobic digestion reactors, the microbial equilibrium that yields the best performance in these reactors has only recently been hypothesised. In this study, two full-scale continuous anaerobic reactors, placed in Torino's main wastewater treatment plant in northern Italy, were followed to develop a summary indicator for measuring anaerobic digestion performance. A total of 100 sludge samples were collected. The samples were characterised chemically and physically, and microbial groups were quantified by qRT-PCR. A chemical biological performance index strictly correlated to specific biogas production (rho = 0.739, $p < 0.01$) is proposed. This approach will produce new management tools for anaerobic digestion in wastewater treatment plants.

1. INTRODUCTION

Across the world, energy production and consumption are of utmost concern in environmental sustainability strategies. As part of the effort to alleviate worldwide energy problems, a green economy has grown rapidly. In Europe, annual energy production from 2000 to 2011 increased from 400,000 GW/h to 680,000 GW/h, of which 17% was derived from biomass and biogas. In 2011, the growth rate of electricity produced from biogas was 18.2% (EUROSTAT, 2012). In Italy, biogas energy comes from landfills (82%), municipal biogas plants (17%) and wastewater treatment plants (WWTPs) and accounts for only 1% of total electricity production (Bodik et al., 2011). Biogas production from scrap biomass reduces the environmental impact of waste disposal, in terms of organic carbon, pathogen and some toxicant reduction. Today, biogas is the most common form of energy recovered from wastewater; moreover the potential energy recovery

70 from WWTP is approximately 8 KJ of electricity and 17 KJ of heat per household, using an average
71 of 380 L water/day (Elias-Maxil et al., 2014). The advantage of biogas production is conditioned by
72 a wide range of management variables, including the biomass sources used for biogas production,
73 with particular regards to the co-digestion configuration in which biomasses coming from different
74 origins were included, the capability to produce biogas in large quantities in a full-scale plant
75 (Jenicek et al., 2013), and the ability to use produced energy (heat, steam, electricity, and
76 hydrogen) in different applications. After biogas production, the digested sludge volume can be
77 reduced up to 60%, including the digested sludge end treatment; on the other hand, AD also has
78 an environmental impact that must be evaluated and considered. In Italy, biogas energy accounts
79 for one-third of the renewable energy produced (28,000 GWh) but remains at only 20% of the
80 estimated potential production (Pignatelli et al., 2012). Despite the increase in renewable energy
81 production, economic activity in this sector has declined in Europe over the last few years (-11
82 billion euro, -7.8%, from 2011 to 2012). An analysis of the situation indicates a need for tight
83 interactions between businesses, private companies and public institutions to define real needs
84 and an efficient pathway to expand the green economy and renewable energy production.
85 Environmental sustainability is obviously hindered by economic sustainability, as economic
86 sustainability is influenced by the yield production efficiency. For this reason, optimisation
87 methods ranging from the treatment of input biomass (Ariunbaatar et al., 2014) to the elaboration
88 of mathematical models (Donoso-Bravo et al., 2011) have been widely proposed in the literature.
89 AD is intrinsically a multi-step chemical and biochemical process, and many factors (e.g.,
90 microbiological, operational, and chemical) can affect AD performance. During AD,
91 microorganisms living in a reducing environment can use organic matter for their fermentative
92 metabolic processes (Diaz et al., 2011). The high complexity of AD may lead to many serious
93 problems (such as instability, long retention times, low efficiency, and highly polluted supernatant)

that prevent AD from being adequately controlled and widely commercialised (Mata-Alvarez et al., 2011). Anaerobic digestion is primarily a microbiological process, so chemical physical indicators that are able to condition microbial growth and the microbiological composition of the microbiota in digesters have been widely studied and published (Cardinali-Rezende et al., 2012; Koch et al., 2014). A wide diversity of microbes participates in the microbial food chain, gradually degrading complex molecules to a mixture of essentially methane (CH_4) and carbon dioxide (CO_2). In fact, more than 1,000 representatives of the Bacteria domain have been identified by metagenomic studies (Wirth et al., 2012). The majority of the species that have been identified in biogas reactors are members of the Clostridia and Bacilli classes, along with members of the Bacteroidia, Mollicutes, Gammaproteobacteria and Actinobacteria classes. Among the Archaea, the most abundant acetotrophs are Methanosarcina spp., while the most abundant hydrogenotrophs are Methanoculleus spp., Methanospirillum spp. and Methanocorpusculum spp. (Cardinali-Rezende et al., 2012; Wirth et al., 2012). A clear understanding of the organisation and behaviour of this complex community is crucial for optimising performance and attaining a stably operating process. Only recently have some microbiologic indicators of good AD performance been proposed, such as Methanosarcina spp. and syntrophic acetogenic bacteria (De Vrieze et al., 2012). However, a model useful for describing WWPT-AD performance has still not been assessed. Studies on lab-scale digestions are widely different from real industrial scales, and observations of lab-scale digestions must be verified in operative industrial plants. The quantity and quality of the biogas produced are important criteria, especially when technologies for biogas valorisation, such as fuel cells or vehicle traction, are introduced.

The presence of trace amounts of undesirable substances such as hydrogen sulphide (H_2S), siloxanes (SiO-R2), halogens (Cl), and mercaptans (CH_3SH) in the raw biogas could be dangerous to the equipment used in energy valorisation. In particular, H_2S is a toxic compound, and the

oxidation products of H_2S formed during combustion are highly soluble. During AD, H_2S is produced by sulphate-reducing bacteria, and such microorganisms have been widely studied by biomolecular techniques in the last decades (Muyzer and Stams, 2008). The aim of this work is to perform a chemical and biological characterisation of the sludges and to develop an indicator parameter that includes microbiological metrics for use in optimising the quantity and quality of biogas produced by AD in WWTPs. Moreover, a synthetic biological and chemical performance index for this technology is proposed.

2. MATERIALS AND METHODS

2.1 WWTP description

The plant considered in this work is a WWTP in Castiglione Torinese (Italy) belonging to the SMAT (Società Metropolitana Acque Torino) S.p.A. group. This plant treats wastewater from the Torino metropolitan area. This plant is the largest WWTP in Italy, serving over 2 million population equivalents in the Turin metropolitan area and treating over 620,000 cubic metres of wastewater daily. The treatment plant operates on two treatment lines. The first line treats water through 4 parallel modules, where water is purified by a chemical-physical-biological process; the second line is able to treat the sludge produced by water treatment. In the second line, sludges are thickened, biologically stabilised (partially converting its organic content to biogas and then into electrical and thermic power), and then dewatered before disposal.

Primary wastewater treatment is designed to remove particles with settling rates of 0.3-0.7 mm/s. At the end of the process, the degraded primary sludge is pumped to the plant's sludge handling facilities for further processing, and the partially treated wastewater from the primary settling tanks flows to the secondary treatment system. This treatment includes pumping air into the sludge to facilitate further settlement of particles. Some of this settled sludge is circulated

back to the aeration tanks to stimulate an activated sludge process. The recirculated sludge contains an enormous number of microorganisms that help to maintain the right mix of bacteria and air in the tank and also facilitate the removal of as many pollutants as possible. The remaining secondary sludge is removed from the settling tanks and added to the primary sludge for further processing in anaerobic reactors (mixed sludges). All sludge types (primary sludge and mixed sludge) coming from the water line undergo several processes devoted to biological stabilisation and volume reduction. The primary aim of AD is to transform most of the organic content in thickened sludge into biogas. This process takes place in 6 anaerobic digesters (12,000 m^3 each) configured to operate continuously. Sludge is stored with a mean hydraulic retention time of approximately 20 days under mesophilic conditions. After anaerobic digestion, the sludge is heat dried, evaporating all the water from the sludge. The dried sludge is then recovered in condensers with thermal recovery. Sludge exiting this phase is almost dry, containing less than 10% water. Before energy recovery, the biogas produced during anaerobic digestion is stored in three gasometers with a total volume of approximately 16,900 m^3.

2.2 Sampling collection

One hundred samples were collected over the course of a year (13 February 2012 to 28 January 2013). Every 15 days, samples were taken from two different anaerobic digesters. The first biodigester is fed with sludge from secondary wastewater treatment (2061 and 3034 samples), while the second biodigester is fed with mixed sludge (50% sludge from primary treatment + 50% sludge from secondary treatment; 2058 and 3033 samples). The 2061 and 2058 samples were collected from the influent (feeding input sludge), and the 3034 and 3033 samples were collected from the effluent (digested output sludge) of each biodigester (Figure 1). The hydraulic retention times for the first and second biodigesters were 23 ± 9 and 25 ± 13 days, while the process

166 temperatures were 33 ± 15 and 32 ± 13 °C for the 3033 and 3034 samples, respectively. For each
167 sample type (2058, 2061, 3034 and 3033), 25 samples were collected. Each sample consisted of
168 500 ml sludge collected in sterile PET bottles intended for microbiological analysis. Chemical and
169 biological analyses were performed on these samples.
170
171 **2.3 Chemical analysis**
172 Chemical analyses were performed by the SMAT Laboratory, which has been accredited in
173 accordance with ISO 17025, 2005. This laboratory performed compliant testing on sewage waters,
174 applying additional precautions to the sludge matrix treatment not covered by the ISO standard.
175 Chemical Oxygen Demand (COD) analyses of sludge samples were performed in accordance with
176 ISO Certification (ISO 15702, 2002). The pH values of the liquid matrices were measured
177 potentiometrically in accordance with CNR IRSA method ((ISO-compliant test) using a portable
178 WTW pH metre – pH315i and WTW pH-electrode Sentix-41. APHA Standard Methods for the
179 Examination of Water and Wastewater (APHA, 2012) were used in the gravimetric determination
180 of Total Suspended Solids (TSS) and Volatile Suspended Solid (VSS) in sludge samples. Sulphate
181 (SO_4^{2-}) in sludges was measured via liquid/solid extraction and ionic chromatography in
182 accordance with the APHA Standard Methods for the Examination of Water and Wastewater
183 (APHA, 2012). The following equipment was used for chromatography: Ionic Chromatography
184 mod. ICS 3000 – Dionex, column AS4A-SC 4X250 mm – Dionex p/n 043174, pre-column AG4A-SC 4
185 mm – Dionex p/n 043175, suppressor ASRS 300 4 mm – Dionex p/n 064554, and autosampler AS-
186 DV- Dionex p/n 068907. Internally validated SMAT methods were used for the elemental analysis
187 of hydrogen (H), nitrogen (N) and sulphur (S) with an electronic MX5 Micro Balance (resolution=1
188 µg, and unit=6 digit) by Mettler Toledo and a THERMO FlashEA 1112 Series Elemental Analyser.
189

2.4 Biogas analysis

The SMAT Laboratory analysed the chemical composition of the raw biogas and pollutants generated by anaerobic digestion. The SMAT Laboratory performed samplings and analysis of methane, hydrogen sulphide, and carbon dioxide. Sampling was carried out by filling gas sampling bags. In the laboratory, gas bags were connected to a micro GC that allows for separation into different elements. A 2-channel micro (CP 4900 Varian) was configured with a 5 Å CP-MolSieve, PPU columns, and a Thermal Conductivity Detector (TCD). Analysis of other pollutants, including halogen compounds, halocarbons and siloxanes, was performed by a certified external laboratory with internally accredited methods.

2.5 DNA extraction

Fifty microlitres of fresh sludge from each biodigester was centrifuged at 4000 g for 10 minutes, and the supernatant was discarded. The pellet was dried overnight at ambient temperature. DNA extraction was performed with a commercial kit (PowerSoil DNA Isolation Kit, MO-BIO Laboratories Inc., Carlsbad, CA). According to the manufacturer's instructions, the 0.25 g semi-dry pellet was subjected to a number of steps to break down the row matrix, facilitating DNA release. Several steps of DNA purification were then performed. DNA was finally separated with column tubes and resuspended to a 100 µl stock volume. For each sample, DNA extraction was performed in triplicate. Fluorimetric quantification of each DNA sample was performed using a Qubit™ Fluorometer and the Qubit™ dsDNA HS Assay by Invitrogen (distributed by Life Technologies Ltd. – Paisley, UK) according to the manufacturer's instructions. Samples were stored at -20°C prior to PCR analysis. The average extracted DNA concentration was 48.16 ± 33.30 µg/ml; the level was highly variable but conformed to other levels reported in the literature for sludges. The amount of DNA extracted from the secondary treatment samples, which are normally richer in aerobic

214 microorganisms, was higher (50 samples from mixed system: 32.0 µg/ml *vs* 50 samples from
215 secondary system: 64.3 µg/ml; T-test: <0.0001).
216
217 **2.6 RT-qPCR**
218 All samples were tested in triplicate, and DNA quality and integrity were evaluated by gel
219 electrophoresis prior to the PCR analysis. Real-Time Quantitative PCR (RT-qPCR) was performed
220 using a Chromo4 thermal cycler (Bio-Rad – Hercules, CA) and Opticon Monitor 3 Software. The
221 amplification target for total bacteria quantification (TotBact) was the ribosomal RNA 16S subunit
222 (*16sRNA*) (Dridi et al., 2009), for methanogen quantification (MN), the functional gene methyl-
223 coenzyme M reductase α-subunit (*mcrA*) (Steinberg and Regan, 2008), and for sulphate-reducing
224 bacteria quantification (SRB), the a-subunit of dissimilatory APS reductase (*aprA*) (Meyer and
225 Kuever, 2007). For MN and SRB, 2 µl of tenfold diluted samples was added to the reaction
226 mixtures consisting of 10 µl SsoFast EvaGreen® Supermix (Bio-Rad – Hercules, CA), 0.5 µl each of
227 the forward and reverse primers (10 µM concentration, Thermo Fisher Scientific, Waltham – MA)
228 and 7 µl of ultrapure water in a 20 µl final reaction volume. For TotBact and all methanogen
229 targets (*Methanosarcina* - msar*, Methanocorpusculum* - mcp*, Methanospirillum* - msp and
230 Methanobacteriaceae - *mbac*), 2 µl of the tenfold diluted samples was added to reaction mixture
231 consisting of 8 µl IQ™ Multiplex PowerMix (Bio-Rad – Hercules, CA), 0.2 µl molecular probe (10
232 µM concentration), 0.5 µl each of the forward and reverse primers (10 µM final concentration,
233 Thermo Fisher Scientific, Waltham – MA) and 8.8 µl of ultrapure water in a 20 µl final reaction
234 volume. The primers used for methanogen families were the same as for MN, including specific
235 probes (Steinberg and Regan, 2009). To obtain an absolute quantification of all targets in the
236 sludge samples, the genomic DNA of each microorganism, provided by the American Type Culture
237 Collection - LGC (ATCC – Manassas, VA), was used as the standards. Serial tenfold dilutions of each

238 ATCC standard were assayed, and quantifications are expressed as the gene copy number/µl of
239 extracted DNA, assuming four 16Srna gene copies *per* bacterium (Merlino et al., 2012). The total
240 MN was quantified using a standard curve in which the *mcrA* gene from *Methanosarcina*
241 *acetivorans* was placed into the pCR21 vector (Steinberg and Regan, 2008). TOP10 *E. coli* cells
242 were transformed with the *mcrA* plasmid to amplify the plasmid, and the plasmid was then
243 extracted using a commercial kit (NucleoSpin Plasmid – Macherey-Nagel, Düren, Germany). A
244 tenfold standard curve was determined as previously described (Traversi et al., 2012). Table 1
245 provides detailed information regarding the sequences and standard genomic DNA used in the
246 PCR analyses. For MN and SRB, the reaction conditions were 95 °C for 3 min (1X), and then 95 °C
247 for 3 sec, 55 °C for 45 sec, 72 °C for 30 sec and 83 °C for 5 sec (40X). A final melt curve analysis was
248 performed to verify the specificity of the PCR products. The melt curve program was as follows:
249 denaturation for 1 min at 95 °C, cooling for 1 min at 65 °C and then heating to 95 °C at a rate of
250 0.5°C per cycle. For methanogen groups and TotBact, the reaction conditions were 95 °C for 3 min
251 (1X), then 95 °C for 30 sec, 55°C for 1 min (39X). The other amplifications were performed for 30
252 sec, 55 °C for 1 min (40X); a melt curve was not performed. To confirm the amplification of each
253 target, gel electrophoresis was performed on 2% agarose gels, and the size of each fragment was
254 compared with the literature. Finally, triplicate averages were accepted only when the coefficient
255 of variation was below 20%. The reaction efficiency was accounted for in all PCRs (Table 1).
256
257 **2.7 Statistics**
258 Statistical analyses were performed using the SPSS Package, version 21.0. The following methods
259 were applied: (1) a log transformation of non-normally distributed data; (2) the Pearson rank-
260 order correlation coefficient to assess relationships between variables; (3) a T-test to compare
261 means; and (4) an ANOVA for multivariate analysis, in which an equal variance was assumed,

followed by a Tukey post-hoc test for multiple comparisons; and (5) a multivariate linear regression model by blocks specifying the dependent variable and the assumed predictors. The mean differences and correlations were considered significant if $p<0.05$ and highly significant if $p<0.01$.

3 RESULTS AND DISCUSSION

3.1 Chemical analysis of the feedings

Table 2 provides a descriptive analysis of the chemical parameters detected in the two anaerobic systems, both for input sludge (columns 2 and 5) and for output sludge (columns 3 and 6). Additionally, the ANOVA results comparing the four data series and T-test results considering input samples versus output samples for each digester are reported in Table 2 (column 9, 4, and 7, respectively). The feeding quantity was variable (from 5.544 to 29.821 m^3/month) for both digesters and was higher during the first sampling period, especially for the mixed sludge digester. Mean monthly feeding volumes during the first semester were 22.095 ± 2.183 m^3 and 20.457 ± 5.580 m^3 for the 3033 and 3034 digesters, respectively. Mean feeding volumes during the second semester were 12.882 ± 4.518 m^3 and 14.195 ± 5.312 m^3, respectively. Various physical and chemical differences were observed in the feedings due to the different types of sludge introduced. The TSS% and VSS% were higher in the mixed sludge compared to the secondary sludge (Table 2), but the differences are not significant. The ratio between VSS and TSS was 0.71 for both feedings.

There is a significant difference between the total and organic carbon input between the two systems (Figure 2A, $p < 0.0001$): the mixed sludge is richer in carbon. In secondary treatment, a portion of the organic carbon is degraded by the aerobic population. Because part of the mixed sludge is not subject to secondary treatment, more carbon is available for digestion by anaerobes.

286 This result is supported by the significant difference observed (p<0.0001) in the CODs of sludge fed
287 to the digesters (Table 2). The pH of the mixed input is lower due to the influence of the primary
288 sludge; the acidity in the mixed sludge is also markedly higher (an order of magnitude, p<0.01)
289 (Table 2) and such difference was confirmed in other studies (Huang and Wang, 2014). This
290 feeding alkalinity is too low, as alkalinity levels above 1000 mg/L are generally better for anaerobic
291 digestion processes (Amani et al., 2010). Hydrogen was slightly higher in the mixed sludge input
292 compared to secondary sludge, but this difference is not significant. Nitrogen was lower in the
293 mixed sludge compared to secondary sludge (T-test p=0.023). Elemental sulphur is not significantly
294 different between the two sludge feeds. However, the chemical species of sulphur that were
295 present differed. The main sulphur compounds were sulphates in both feeds, but sulphate was
296 particularly concentrated in the secondary sludge (p < 0.0001). Sulphite and sulphide were also
297 present in the mixed sludge (56% and 8% of the sulphate level, respectively) (Table 2). Particular
298 attention should be paid to this sulphur species trend, as the trend may indicate the possible
299 presence of the sulphur compounds in the raw biogas and in specific treatment digestion lines.
300 The COD/SO_4^{2-} ratio is an important parameter that indicates the equilibrium between organic
301 matter and sulphur species. A high COD/SO_4^{2-} ratio at the laboratory scale has been proven to
302 benefit the production of large amounts of biogas in terms of methane concentrations because
303 the acetate was used by methanogens in competition with sulphate reducing bacteria. Conversely,
304 low COD/SO_4^{2-} ratios result in a biogas composition with higher sulphur compounds (Moon et al.,
305 2013). The COD/SO_4^{2-} ratio reported in the literature, determined only at the laboratory scale to
306 test the effect of a great amount of sulphate as a cut-off for methanogen inhibition in favour of
307 sulphate reducing bacteria, is equal to 3, an increase in magnitude of four orders from this study
308 data (Figure 2B). The COD/SO_4^{2-} ratio is approximately fivefold higher in the mixed input system
309 compared to the secondary input system (p=0,006) due to the higher COD and lower sulphate

level in the mixed system. This ratio refers only to dissolved sulphates but is presumably proportional to total sulphate levels. Moreover, only free sulphates are likely bioavailable for SRB (Barrera et al., 2014).

3.2 Chemical analyses of the outputs

Table 2 provides a descriptive analysis of the chemical parameters detected in samples of sludge inside the continuous digesters (column 3 and 6). Figure 2A clearly illustrates organic removal by the two anaerobic processes. For mixed sludge, organic removal is higher. Expressed as a percentage of organic carbon, the removal from mixed sludge was 20%, while removal from secondary sludge was 5%, a statistically significant difference. Additionally, the TSS, VSS, TSS/VSS and COD decreased in the bioreactor fed with mixed sludge (Table 2). In the 3034 digester, which was fed with secondary sludge, organic removal was less clear, as differences in the TSS, VSS and VSS/TSS and COD of 2061 *versus* 3034 were not statistically significant. The literature reports that introducing a greater amount of secondary treatment sludge into the primary sludge results in a VSS increase in the output sludge. As the amount of secondary sludge is increased, the amount of VSS increases, suggesting a different VSS composition following secondary treatment (Rozzi, 1988). The best COD removing efficiency observed (for mixed sludge) was approximately 50%, which, so far, does not agree with data reported by Zhang, which shows a removal efficiency of approximately 65-70% (Zhang et al., 2013). The initial COD levels can vary over a wide range, which influences the removal. In optimised systems, the COD removal efficiency generally can be maintained above 80% (Hutnan et al., 2013).

The hydrogen percentage was found to be significantly different between the sludge fed and the sludge inside the digester for both the mixed and secondary systems (Table 2). Hydrogen decreases significantly during the anaerobic process, and a higher difference was observed for the

334 mixed system (-19%). Similar behaviour was observed for nitrogen, but the differences were not
335 significant. In both systems, the output pH is neutral. MN are extremely sensitive to pH; however,
336 fermentative microorganisms are in general somewhat less sensitive and can function in a wider
337 pH range (between 4.0 and 8.5) (Liu and Whitman, 2008).
338 Elemental sulphur, expressed as a percentage of total solids, is higher in the output samples, but
339 this result is only statistically significant for the mixed sludge (T test p<0.0001). The observed
340 increase can be explained by the significant portion (approximately 50%) of total solids removed
341 by gasification and the fact that little elemental sulphur was lost in the biogas. This leads to a
342 marked decrease in the denominator and a consequent increase in the percentage. Sulphates, as
343 useful substrates for the SRB, were decreased in both systems, but the difference was significant
344 only for the secondary sludge system, where sulphates in the input were higher.
345 The secondary system shows a higher sulphate consumption compared to the mixed system (-
346 77%, p<0,001; -39% not significant); on the other hand, the sulphate concentration in the
347 secondary sludge was minimally at least three times higher than that in the mixed sludge.
348
349 **3.3 Microbial analysis**
350 The microbial characterisation results for both the input sludge (columns 2 and 5) and the output
351 sludge (columns 3 and 6) from the two anaerobic systems are summarised in Table 3. In addition,
352 ANOVA results comparing the four data series and the T-test results comparing the input samples
353 to the output samples for each digester are reported (columns 9, 4, and 7).
354 The absolute quantifications of various microorganism groups, including TotBact, SRB, and MN, as
355 well as 4 different methanogen groups (msar, mcp, msp, mbac), are shown. It was not possible to
356 calculate the min, max and SD values for msp, as this family was often not present in the samples
357 investigated. The msp concentration in many samples was below the limit of quantification. More

358 specifically, the percentages of samples under the limit of quantification were 20% for msar, 24%
359 for mcp, 92% for mbac, and 96% for msp.
360 TotBact concentrations were always higher than both MN and SRB, and MN concentrations were
361 higher in the outlet samples compared to the input samples, as expected (Figure 3). These
362 differences were statistically significant (ANOVA p<0,0001 for all comparisons) (Table 3). In this
363 study, a significant seasonal trend was not observed. In fact, the parameters found to affect levels
364 of microbiological groups were not linked to a particular seasonal variable (e.g., temperature).
365 Also, although mesophilic conditions were maintained, quantities of selected parameters carried
366 out from the beginning of the digestion process (e.g., msar, mcp and MN) changed markedly
367 between the 16° sampling and the end (Figure 3). Notably,, the feeding quantity decreased after
368 August and decreased markedly after October to the end of the sampling (Figure 4). As a result, an
369 increase in methanogens not belonging to the families investigated was observed (Figure 3). In
370 general, the amount of microorganisms in the feedings affects the concentration of
371 microorganisms quantified in the digester.
372 TotBact levels were higher in the secondary system than in the mixed system, as shown in Table 3.
373 This difference is statistically significant (p<0,05). A T-test comparing input and output samples
374 from the same digester indicates a significant difference only for secondary sludge (Table 3). The
375 TotBact decrease observed during this process shows that the population was subjected to
376 anaerobic selection. Additionally, this system showed low TotBact and SRB variability compared to
377 the mixed system, where different samples showed lower concentrations (Figure 3 for samples 1,
378 4, 9, 13 and 15). A trend similar to that observed for SRB was observed for TotBact (Figure 3), with
379 a higher SRB level in the secondary sludge before digestion (Table 3). Digestion of the mixed
380 sludge is able to limit SRB growth compared to secondary sludge. A T-test comparing input and
381 output samples from the same digester shows a significant difference for both the mixed sludge

382 and the secondary sludge (table 3). MN concentrations are quite similar in the two systems.
383 Output MN levels are clearly higher, indicating the presence of an anaerobic selective pressure
384 during digestion (Figure 4). A T-test comparing input and output samples from the same digester
385 shows a significant difference both for the mixed sludge and the secondary sludge (Table 3). In the
386 first sampling period, the MN population consisted almost entirely of msar, especially in the input
387 samples. Other methanogens became prevalent in the output samples after AD selection (Figure
388 3). As highlighted in Figure 3, MN levels vary along the sampling period, increasing from the
389 beginning of the sampling until the end. As discussed previously, this increase is not attributable to
390 seasonal influences, as sampling began and ended in the same period of the year. Instead, this
391 increase is due to WWPT management. Total MN levels were inversely correlated to feeding
392 quantity expressed as mass of the VSS fed (rho = -0.545, p<0.01), while msar and mcp levels were
393 directly correlated to the feeding quantity (rho =0.538, p<0.01 and rho = 0.422, p<0.01). These
394 correlations suggest that methanogens that predominantly use acetate as a substrate flourish
395 when a large quantity of VSS is fed, a behaviour that has been reported in the literature (De Vrieze
396 et al., 2012). Conversely, acetate deficiency is correlated with selection for other methanogens
397 (Kotsyurbenko et al., 2007; Lee et al., 2014).
398 In the present study, by using a single genera/family quantification approach instead of a total
399 characterisation approach, only a fraction of the methanogen population was described. The
400 fraction described was very variable and ranged between 1% and 100%, with an average value of
401 40% (Figure 3). The uncharacterised fraction was more prevalent in the output samples and after
402 the middle of the sampling period (i.e., after the beginning of August). Among the quantified
403 methanogens, the main populations detected were msar and mcp (Figure 4). The msar genus was
404 detected in both the input and output samples (Figure 4). However, no selection for this genus
405 was present during digestion. In fact, neither the ANOVA model nor the T-test comparing input

406 and output samples revealed any significant differences. In most of the literature regarding
407 digestion characterisation, msar selection is reported (De Vrieze et al., 2012).This genus seems to
408 be prevalent only for the digestion period prior to the decrease in feeding. As described
409 previously, this feeding decrease or other process variation likely affected the msar presence.
410 After msar, mcp is the second most prevalent genus of the genera evaluated in the digesters,
411 especially during the second part of the sampling period. No significant differences were detected
412 in mcp concentrations between input and output samples, and the measured concentration was
413 approximately 3 log for each µl of extract. As for correlations between microbial communities, a
414 high and significant correlation between TotBact and SRB levels and TotBact and MN levels were
415 observed. In addition, MN levels are correlated with SRB levels, a correlation that can be explained
416 by the ability of both MN and SRB to use acetate and hydrogen (Guerrero et al., 2013) (Table 4).
417
418 **3.4 Chemical-biological correlations**
419 TotBact levels were directly and significantly correlated with the amount of total solids introduced
420 into the digesters (Pearson's coefficient**:** 0.203, $p<0.05$). An opposite and significant correlation
421 between TotBact and sulphide was observed in such systems. This correlation could be explained
422 by dissolved hydrogen sulphide's general toxic effect on microbial populations (Guerrero et al.,
423 2013), even if no confirming kinetic study was performed.
424 MN levels were inversely correlated with temperature (Pearson's coefficient: -0.329, $p<0.05$). On
425 average, the temperature was 32.7 ± 14.1 °C, so a selective pressure for methanogens with
426 optimal growth temperatures below 30°C appears to be present (Lee et al., 2014). Levels of
427 methanogens with higher optimal growth temperatures (~37°C), such as msar (Liu and Whitman,
428 2008), were directly correlated with temperature (Pearson's coefficient: 0.316, $p<0.01$). The
429 literature reports a marked shift from acetoclastic to H_2-dependent methanogenesis at low

acidities (below pH 4) and low environmental temperatures (Kotsyurbenko et al., 2007). In the studied reactors, the pH and temperature conditions were not so extreme, but a partial shift resulting in the lowering of these process parameters was possible. A negative and significant correlation was observed between the MN levels and the various chemical parameters describing the organic content of the matrix, including total carbon (Pearson's coefficient: -0.267, $p<0.01$), organic carbon (Pearson's coefficient: -0.381, $p<0.01$), and VSS (Pearson's coefficient: -0.244, $p<0.05$). In this continuous system, bioavailable organic content is used in MN metabolism. So this is explained by the ability of the methanogens, in anaerobic conditions, to transform the organic matter in methane, removing such elements from the sludge. While the total and organic carbon was not significantly correlated with the total bacteria.

Additionally, an inverse correlation between the elementary hydrogen and MN levels was observed (Pearson's coefficient: -0.310, $p<0.05$), indicating that hydrogen was consumed by the methanogens, likely through H_2 and formic acid. MN levels were directly correlated to the pH (Pearson's coefficient: 0.216, $p<0.05$) and alkalinity (Pearson's coefficient: 0.229, $p<0.05$). According to the literature, MN is vulnerable to environmental acids and requires a higher alkalinity (Amani et al., 2010). Mcp levels were also directly correlated with alkalinity (Pearson's coefficient: 0.264, $p<0.01$). Finally, an inverse correlation was observed between the elementary nitrogen and MN levels (Pearson's coefficient: -0.220, $p<0.05$); this correlation could be related to a decrease in organic content and to an increase in gas species, in particular ammonia and molecular nitrogen, which remain untransformed by anaerobic digestion. A direct correlation between elementary sulphur and mcp levels was observed (Pearson's coefficient: 0.307, $p<0.01$). Unlike other methanogens and SRB, this last group of methanogens is unable to use acetate, a trait that could explain this direct correlation.

Msp and mbac levels do not correlate significantly with any chemical parameters; however, this may be due to the low concentration and variability of these families in the biodigesters.

The microbial quantification of SRB highlights the reduction pathway for sulphate consumption in SRBs. Globally, following the in-out matter flux, sulphide and total sulphur show increasing concentrations; conversely, sulphite and sulphate compounds show the reverse of this trend (Table 2). These results, also reinforced by microbial quantification of SRB, highlight the dissimilatory reduction pathway in which sulphate represents the substrate for SRB. The correlation between SRB and sulphate levels ($rho = 0.252$, $p<0.05$) indicates that the presence of SRB is sulphate-dependent. The presence of SRBs is inversely correlated to sulphide and sulphite ($rho = -0.496$, $p < 0.01$ and $rho = -0.217$ $p < 0.05$, respectively) and it results in gradual transformation of sulphate at a rate influenced by the input/output matter flux. The pH measurements fell within a range of values (below 6 pH) that promote sulphide volatilisation. Additionally, a significant correlation between SRB levels and nitrogen levels in the samples was observed (0.307, p=0.002). This correlation could be explained by the ability of some SRBs to fix nitrogen (Rabus, 2006).

3.5 Systems comparison and performance index elaboration

Figure 4 depicts the total cubic metres of biogas produced in each digester over the sampling year for mixed sludge (Figure 4A) and secondary sludge (Figure 4B). These figures also show the amount of VSS fed monthly to the digesters and the methanogen quantities measured in samples collected from the digesters on particular dates. Considering the substantial similarity between the two anaerobic digesters, it is notable that the mixed sludge system produces a greater amount of biogas (2.158.641 m^3 for the mixed sludge system *versus* 1.448.800 m^3 for the secondary sludge).

The cumulated biogas production shows a significant correlation only with msar (rho = 0.338 $p<0.05$); moreover, such raw relations do not take into account the amount of introduced feedings. So this is only a raw observation, and the details of the different metrics need to be discussed completely, as shown in table 5 in the box on biogas quality. Clearly, the small amount of VSS introduced and removed from secondary sludge during digestion strongly affected the biogas production rate. The efficiency of biogas and methane production was confirmed by correlating the specific production rate with the amounts of VSS added and removed (Table 5). The microbiological communities in the digesters varied mainly as a function of the organic load, a variation that was also observed in the studied case. From the beginning of August, there was an evident decrease in the amount of VSS fed, especially for the mixed system; this decrease was accompanied by a decrease in the amount of biogas produced. It is clear that the msar genus is replaced by other methanogen families not investigated in this study. These methanogen families likely have affinities for lower organic loads, such as *Methanosaeta* (De Vrieze et al., 2012). In the digester fed the secondary sludge, the decreased biogas production corresponds with the reduced msar levels, while the MN levels remain high. The methanogen selection pressure has therefore shifted towards different methanogen groups that are likely less efficient for biogas production (Figure 3).

The methane percentage in the biogas is quite constant, as is the concentration of H_2S in both systems. The microbiological variations related to CH_4 (%) and H_2S (ppm) are shown in Table 5. This table also contains data describing the performance of the two anaerobic digesters, for microbial growth and selection and biogas production in terms of quantity and quality. The microbial data were aggregated to calculate significant ratios, dividing the abundance of different microbial groups, as described previously in the literature (De Vrieze et al., 2012). This type of approach is useful to determine benchmark values for each ratio. For example, a value greater

than 0.1 is significant for the MN/TotBact ratio (Yan, 2013). A value between 0.2 and 0.5 could be considered optimal, an elevated proportion (tending to at least 0.5) of msar and mcp is auspicial and a low presence of SRB in relation to TotBact and MN is most favourable; however, the utility of such benchmarks are unclear at the moment, and further microbial characterisation of anaerobic processes is necessary to elucidate meaningful relationships. Regardless of utility, ratios for the two systems can be compared, and the observed ratios were better for 3033 compared to 3034. MN/TotBact was higher (T-test $p<0.01$), SRB/TotBact was lower (T-test $p<0.05$), and SRB/MN was lower as a result (T-test $p<0.001$). Msar/MN and mcp/MN were not significantly different. Significant production in the 3033 system was observed in terms of the total biogas production, mean daily production, and amount of VSS removed from the feed. Additionally, methane production by the 3033 digester was better than that by the 3034 digester. In terms of biogas contaminants, significant homogeneity was observed in the H_2S contaminant and variable trace contaminant values. More specifically, lower halogen concentrations but higher siloxane and halocarbon concentrations were observed in the 3033 system compared with the 3034 system. However, these differences were not statistically significant. Such information regarding contaminants is important for the biogas storage and energetic valorisation, especially when new methods were included such as fuel cells. The following discussion is focused only on the 3033 mixed sludge feeding for two reasons. First, analysis of the data in Table 5 suggested that the 3033 system performed better than the 3034 system. Second, the mixed sludge feed is more representative of real feeds to WWTP anaerobic digesters. The multivariate regression is highly significant when biogas production during the period between consecutive samplings is considered as the dependent variable, and organic carbon, acidity, alkalinity and volume of feedings to the digester as predictors ($p<0.0001$; volume of feeding $p<0.01$, Beta=0.701; organic carbon $p=0.013$, Beta=0.454). In addition, multivariate regression considering biogas production

525 and methanogen concentrations as predictors showed highly significant results (p=0.016; mcp
526 p=0.016, Beta=0.676; MN p=0.023; Beta= -0.520).
527 Considering this statistical evidence, a synthetic measure (Performance Index) was proposed that
528 can be used to characterise the set of chemical and microbial data. The Performance Index can be
529 calculated using Equation (1) below:
530 (1) PI = (Log mcp)/(Log MN) * %OC * VSSfed
531 where PI is the performance index, Log mcp is the log concentration of mcp, Log MN is the log
532 concentration of MN, %OC is the percentage of organic carbon, and VSSfed is the amount of VSS
533 fed to the digester in tons. PI measures both the prevalent methanogen indicators (as total and
534 mcp concentrations) and the more important chemical parameters in terms of observed impact on
535 the biogas yield. Because it is capable of using chemical indicators determined at the input
536 feedings and microbiologic indicators in the digester to predict the biogas yield, such an index
537 could be used in the management of the process to preview and facilitate an increase in biogas
538 production.
539 This index correlates strongly with the amount of biogas produced, as shown in Figure 5. Hence,
540 the performance index may be used to predict the potential yield of a reactor under a particular
541 set of conditions. The feasibility of such an index is not so difficult and not so expensive. Only the
542 microbiologic parameters are new to the WWPT routine; on the other hand, the PI advantage
543 cannot be assumed to be validated for different WWTP-AD plants. Moreover, anticipation of a
544 decrease in performance by the digestion process could be crucial to AD improvement and
545 management.
546
547 **CONCLUSION**

In closing: the mixed sludge digestion showed slightly better performance in terms of production compared to the secondary sludge; the methods developed are able to describe the microbial equilibrium in the AD for a complete data set; msar is not a good indicator of AD performance in the studied system, whereas mcp seems to be a better bio-indicator and the proposed PI can be a strategic tool for assessing AD production performance. Finally, development of the WWTP-AD needs an integrated approach that includes biological, chemical and technological contributions. Microbial characterisation will reveal the identity of an optimal biogas-producing consortium.

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Table legends:

Table 1: Oligonucleotide primers and probes used in the RT-qPCR analyses.

Table 2: Descriptive analysis of the principal chemical parameters for different samples; numerosity of suitable determination (Num), min and max values, means and standard deviations (SD) are given to characterise the sample distributions of these parameters.

Table 3: Descriptive analysis of the microbial communities for different samples; numerosity (Num), min and max values, means and standard deviations (SD) are given to characterise the sample distributions of these parameters.

Table 4: Correlation between bacterial communities in output samples (N=50); Pearson's coefficients and the level of significance are reported.

Table 5: Descriptive analysis of the two anaerobic digestion systems investigated in this study (3033 and 3034), expressed as microbial group prevalence in the samples and in biogas quantity and quality.

Figure legends

Figure 1: Description of samplings, depiction of sludge origins and the AD configuration in the WWTP.

Figure 2: (A) Organic and total carbon in four sample types and (B) trends for carbon expressed as COD/SO_4 ratios in the two feed types. Dots and whiskers represent mean values and SDs, respectively.

Figure 3: Microbial communities as TotBact, SRB, MN, msar and total detected methanogens – genera and family (msar+mcp+msp+mbac) - in the two treatment sludge lines, subgrouped by feeding (IN samples) and digested sludges (OUT samples). Each concentric circle represents one order of magnitude (as reported in the overlapped scale, from $1{,}0x10^{7}$ to $1{,}0x10^{1}$) in which the microbial communities move; each spoke represents one sample numbered from 1 to 25 according to the sampling schedule.

595 **Figure 4**: Trends in the methanogen community (expressed as total MN, msar and mcp) in relation
596 to the mass of VSS fed (secondary y-axis) and the volume of biogas produced. (A) digested mixed
597 sludges and (B) digested secondary sludges.
598 **Figure 5**: Correlation between the developed Performance Index and biogas quantity from mixed
599 digester 3033 (Pearson's coefficient: 0,739, level of significance <0,0001).
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