Improvement of semi-continuous anaerobic digestion of pre-treated orange peel waste by the combined use of Zero Valent Iron and granular activated carbon

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Abstract

Orange Peel Waste (OPW) is a widely produced residue whose management is complicated by its physical and chemical properties. Anaerobic digestion (AD), which is commonly used for the treatment and exploitation of many biodegradable wastes, is inefficient on OPW due to the presence of essential oils (mainly d-Limonene) as well as the low pH, which cause the process to be instable. Here we explore the effect of alkaline pre-treatment of OPW and of the addition of granular activated carbon (GAC) and Zero Valent Iron (ZVI) in improving AD in two semi-continuous reactors at a laboratory scale. The addition and pre-treatment of ZVI/GAC were shown to help process stability up to a loading of 3 $kgVS \cdot m^{-3} \cdot d^{-1}$ and to increase methane production even at a sub-optimal pH. The investigation of the bacterial community, by high-throughput sequencing, has also increased our insight on their involvement in AD in the presence of ZVI, including its biotic oxidation. In addition, direct interspecies electron transfer was shown to play a role in the reactor supplemented with ZVI.

Keywords: *alkaline pre-treatment, anaerobic digestion, granular activated carbon, orange peel waste, zero valent iron.*

1.1 Introduction

Orange Peel Waste (OPW) is a widely produced residue whose management is complicated by its physical and chemical properties. The major issues behind the management of OPW [1] are linked to the high amount produced, the seasonality of the production, the low pH and high water content, as well as the presence of essential oils (EO) and more specifically of d-Limonene (80-95% of orange EO). A quota of the high amount of OPW produced is presently used as animal feed but, unfortunately, in many parts of the world, uncontrolled dumping near production sites is common with the consequent negative impact on air, water and soil pollution [2].

On the other hand, as demonstrated by several studies [2–6], OPW presents a very high potential as substrate in anaerobic digestion (AD) and, therefore, in terms of biorefining [1] to obtain biofuels and compounds for the chemical, pharmaceutical and food industries.

The problems linked to the toxicity of d-Limonene remain the most significant issue behind OPW management [6]. In fact, until now OPW was managed by AD by applying limited loadings [2] or by co-digestion [7,8]. Research should, therefore, be focused on finding the appropriate pre-treatment methods directed at removing d-Limonene from OPW, thus optimizing its AD and preventing process inhibition. Steam distillation, steam explosion, solvent leaching, biological pre-treatment by fungi, alkali application, ensiling are only some of the techniques, which have been reported in the literature [4,9–11], to have been applied for the removal of d-Limonene from OPW. However, a fully sustainable solution at an industrial scale is yet to be found. Organic matter is used as energy source by heterotrophs' metabolism through processes of oxidation/reduction. However, since solid materials cannot be immediately adsorbed through the cell membrane, two specific metabolic mechanisms have been described, which enable an efficient electron transfer between microbial cells and extracellular solid materials: direct or indirect extracellular electron transfer (EET) [12]. In the

former, metal-containing redox proteins or solid conductive materials are used by microorganisms to electrically connect the intracellular respiratory chain and extracellular solid materials [12]. In the latter, electrons are transferred to or from solid compounds by using diffusible redox chemicals [13].

A particular form of EET is the direct interspecies electron transfer (DIET), which takes place under anaerobic conditions and allows microorganisms to exchange electrons to cooperatively degrade organic compounds (syntrophic metabolism), exchange free electrons and protons or reduce equivalents without using reduced molecules [14], such as molecular hydrogen or formate [15]. Exoelectrogenic and electrotrophic bacteria are responsible for DIET: exoelectrogens export the electrons [16], while electrotrophs accept these electrons to generate their energy [14].

These syntrophic interactions between bacteria and methanogens result in the capacity of the microbial communities to maintain a low hydrogen partial pressure, allowing the system to work efficiently [17].

Therefore, in methanogenic systems, microorganisms can directly transfer electrons to other species by direct electron transfer mediated by conductive materials (CM), such as granular activated carbon (GAC), biochar, carbon nanotubes (CNT) or metal compounds [15]. Electrical microbiologically influenced corrosion (EMIC) is, instead, the phenomenon used to describe the corrosion of iron caused by microbial metabolism via direct uptake of electrons from metallic iron [12] and is reported to enhance methane production during the AD process. As Martins and coauthors [15] report in their review, the advantages of using CM in the AD of different substrates can be summarised, on the one hand, in reducing the lag-phase preceding methane production and, on the other hand, in increasing the methane production rate and organic loading rate (OLR) without affecting COD removal.

However, one of the most used metal-based CM to increase AD is iron (Fe) in its different morphologies and valence states. In fact, the addition of iron facilitates electron transport, stimulates bacterial growth and increases hydrogen and methane production rate by promoting the activity of enzymes [18,19] since most enzymes contain metallic elements, such as Fe and Ni [20].

The corrosion of zero valent iron (ZVI) in aquatic systems under anaerobic conditions is explained by the following oxidation reactions [21,22]:

$$Fe^0 + 2H_2O \rightarrow H_2 + Fe^{2+} + 2OH^- \quad (1)$$

$$8H^+ + 4Fe^0 + SO_4^{2-} \rightarrow S^{2-} + 4Fe^{2+} + 4H_2O \text{ (if sulphur is present)} \quad (2)$$

$$Fe^0 + 2H^+ \rightarrow Fe^{2+} + H_2 \quad (3)$$

The formation of alkaline byproducts and consumption of H^+ stabilizes the pH of the solution, which is a key factor for the growth of microorganisms [21,23–25]. Nevertheless, during the AD of the organic matter, ZVI may increase methane production as it directly serves as an electron donor to reduce CO_2 into CH_4 through autotrophic methanogenesis based on the following reactions [26]:

$$CO_2 + 4Fe^0 + 8H^+ \rightarrow CH_4 + 4Fe^{2+} + 2H_2O \quad (4)$$

$$CO_2 + 4H_2 \rightarrow CH_4 + 2H_2O \quad (5)$$

where H_2 might be produced from the chemical corrosion of ZVI or/and from the hydrolysis/acidification [26]. It has been demonstrated that ZVI may enhance the biological processes utilising hydrogen to decrease the H_2 partial pressure in the biogas, which allow the acetogenesis of fatty acids by hydrogen-consuming microorganisms [27]. Apart from autotrophic microorganisms, CO_2 and H_2 could be used by homoacetogens to decrease their contents in the biogas based on the reaction [26]:

$$2CO_2 + 4H_2 \rightarrow CH_3COOH + 2H_2O \quad (6)$$

In methanogenic environments, DIET has only been demonstrated unequivocally in cocultures of *Geobacter metallireducens* with *Methanosaeta harundinacea* or with *Methanosarcina barke*ri and frequently Geobacter sp. was not detected in improved DIET-driven systems for methane production [15,17,28,29]. Moreover, the detection of the taxonomic composition of the microbial community doesn't seem to directly explain the changes in the mechanisms of interspecies electron transfer. Thus, the improvement of microbial activities in the presence of CM is usually justified by the shift of IET to DIET [15].

As results from the literature, Fe^{II} should be the most suitable Fe valence state to increase hydrogen production compared to ZVI and Fe^{III} [30]. In contrast, concerning the dehydrogenation process, ZVI has a stronger reducing capacity and can act as electron donor [20], reducing the environmental oxidation-reduction potential (ORP), thus stabilizing the pH of the process, increasing the alkalinity by adsorbing CO_2 and promoting enzyme activity and cell synthesis of methanogens [20,31,32]. Moreover, as already previously shown, ZVI is normally oxidised to Fe^{II} [21,22].

In anaerobic digestion, the H_2 partial pressure has to be kept at a very low level in order to enable the process of acidogenesis [33]. Wang and co-workers (2018) observed that the addition of nZVI up to 1.0 $g \cdot L^{-1}$ on AD of waste activated sludge (WAS) improved the production of volatile fatty acids (VFAs), while the further addition of nZVI lead to the inhibition of methanogenesis due to the long-term accumulation of H_2.

On the other hand, in other studies, nZVI acted as an acid buffer enabling the system to maintain the pH at a level between 7 and 8 [35,36], thus increasing methane production and the abundance of syntrophic-methanogenic associations (*Syntrophobacterales* and *Methanosarcinales*) [37].

ZVI has been used as additive in the anaerobic digestion of several substrates. Feng and co-authors [26] observed an increase of protein degradation and VFA production of 22% and 37%,

respectively, in the anaerobic digestion of sludge compared to the control test without the addition of ZVI. The methane concentration increased from 58.5% to 68.9% with the increase of ZVI dosage from 0 $g \cdot L^{-1}$ to 20 $g \cdot L^{-1}$. Finally, the addition of ZVI lead to a change in the microbial community: the abundance of hydrogen-consuming microorganisms including homoacetogens and hydrogenotrophic methanogens with ZVI was higher than that in the control.

Kong and co-authors [38] studied the effect of the addition of ZVI on the anaerobic digestion of the organic fraction of municipal solid waste (OFMSW). An excessive acidification commonly occurs in the conventional AD of this substrate, due to the accumulation of non-acetic VFA that cannot be utilised directly for methane production. Results showed a substantial decrease of total VFA and an increase of over 41% in methane production compared to the test without the addition of ZVI.

Wang and co-authors [21] studied the effects of different ZVI/activated carbon (AC) ratios in the mesophilic anaerobic digestion of sludge. The best results were obtained at the 10:1 ratio: the cumulative methane production was 37.6% higher than the blank and the methane content reached 68.8%. As Martins and co-workers [15] reported in their review, this is a recent research field with a relevant impact in engineered and natural anaerobic microbial processes. The studies available refer to different substrates, different conductive materials and different experimental conditions making comparisons difficult.

External factors can affect the natural selection among microorganisms involved in a specific AD process, leading to different microbial communities [39]. Therefore, to improve the efficiency and stability of the whole system it is important to understand the bacterial lineages catalysing the processes as well as their temporal dynamics. The bacterial community associated to the digested OPW was investigated by amplicon metagenomic analysis of the 16S rRNA gene, in order to study its involvement in the AD with ZVI supplementation.

In this paper the effect of the addition of GAC and ZVI was analysed in two semi-continuous reactors. To the best of our knowledge, this research is unprecedented and is the first attempt to optimise the AD of OPW by using conductive materials with the support of metabarcoding data.

2.1 Materials and Methods

2.1.1 Experimental setup

The experiment was carried out in two reactors (designated as A and B, respectively), which were three-neck bottles with 1.1 L volume (WTW-Germany), equipped with two valves, which allowed the collection of biogas, the feeding of substrate/additives and the withdrawal of sludge. The total operational volume was 600 ml.

Semi-continuous digestion was carried out in mesophilic conditions and for this reason the reactors were placed in a thermostatic cabinet at 35±0.5 °C. The biogas generated was slowly transferred into an alkaline trap (bottle with 1 L of a 3 M NaOH solution) about three times a week by using a 100 mL syringe. In this way the carbon dioxide present in the biogas was absorbed in the alkaline solution while methane caused a pressure increase in the trap and induced the displacement of an equivalent volume of solution [10].

2.1.2 Substrate, inoculum and additives

The substrate used was an OPW collected in an orange transformation industry in Sicily, which was then lyophilised. Lyophilisation has the advantage to preserve the characteristics of the original OPW, allowing, at the same time, to transform the fresh substrate in a fine stable powder, which is easier to feed in semi-continuous reactors. Lyophilised OPW was further subjected to an alkaline pre-treatment using NaOH (5% TS, 50%NaOH solution, Sigma-

Aldrich, St. Louis, MO, USA) at room temperature for 24 h. This process allows to reduce the concentration of d-Limonene and increases the buffering capacity [10].

In the experiment we used an inoculum coming from a local full-scale AD plant, which was mainly fed with manure and various residues from the agro-industry. The inoculum was in the form of a liquid digestate and it was sieved, prior to the experiment, to remove fibrous materials (e.g. straw) and then stored in anaerobic conditions at 35°C to reduce as much as possible the non-specific biogas production.

Substrate and inoculum were characterised in terms of Total Solids (TS), Volatile Solids (VS) and pH according to standard methods [40] (Table 1).

Previous experiments have demonstrated that the C/N ratio of OPW is about 50 and that it is poor in micronutrients; therefore, a nutrient solution is essential to guarantee the stability of the process at a laboratory scale [41]. Thus, solutions, compliant with the UNI/TS 11703:2018 norm, which has recently been introduced in Italy, were periodically added to reduce the C/N ratio and to supply it with micronutrients. The norm includes the use of three different solutions defined as Solutions A, B and C. Solution A contains specified quantities of KH_2PO_4, $Na_2HPO_4 \cdot 12H_2O$, NH_4Cl; the amount to be used was assessed to supplement the nitrogen present in OPW in order to reach a C/N ratio of about 30. Solution B contains $CaCl_2 \cdot 2H_2O$, $MgCl_2 \cdot 6H_2O$, $FeCl_2 \cdot 4H_2O$ and the amount used is the same as Solution A, as stated in the cited norm. Solution C contains $MnCl_2 \cdot 4H_2O$, H_3BO_3, $ZnCl_2$, $CuCl_2$, $Na_2MoO_4 \cdot 2H_2O$, $CoCl_2 \cdot 6H_2O$, $NiCl_2 \cdot 6H_2O$, Na_2SeO_3 and the amount used is 1/5 of the volume of Solution A.

In case of upscaling of the process, it is advisable to substitute nutrient solutions with an appropriate co-substrate.GAC (CARBOSORB 2040 -20 x 40 mesh; Comelt srl, Milan, Italy) was added in both reactors (A and B), whereas ZVI (POMETON srl, Mestre, Italy) was additionally supplied only in reactor B. Following the results of previous findings [41], 20 $g \cdot L^{-}$

^{1} of GAC was added in both reactors at the beginning of the experiment, while the dosage was kept at 0.9 g·d-1 during the rest of the experiment.

As already previously explained, ZVI (POMETON srl, Mestre, Italy) was additionally supplied only in reactor B at a dosage proportional to the substrate fed (0.3 g ZVI·gVS_{added}^{-1} [42,43]). Both additives were fed to the reactors together with the substrate twice per week (usually on Tuesdays and Fridays). By the end of the experiment, the total GAC added to reactors A and B was 46.2 g and 113.5 g, respectively. The total ZVI supplied in reactor B was 57.2 g.

Table 1. Substrate and inoculum characteristics.

	TS (%)	**VS (%TS)**	**pH**	**D-limonene (mg/g)**
Inoculum	4.8	72.8	8.0	-
Raw lyophilised OPW	93.2	96.9	-	0.37*
Pre-treated OPW	12.6*	77.6*	10.7*	0.15*

*Value measured on rehydrated OPW

d-Limonene was extracted from the substrates by mixing 1.5 g of lyophilised sample (eventually pre-treated) with 3 mL of a solution of Toluene (Sigma-Aldrich, St. Louis, MO, USA) and cyclohexane (0.1M, Sigma-Aldrich, St. Louis, MO, USA), which was used as internal standard, for two hours. The liquid obtained was then injected into a gas chromatograph (Agilent 6890) equipped with a wide-bore capillary column and a flame ionization detector (FID). The capillary column was a J&W DB-WAXetr 50 m x 320 mm x 1 mm and the gas carrier was nitrogen with a flow rate of 10 ml/min. The injector was settled at 250°C. The temperature programme started at 50°C, which was held for 8 min; the temperature was then raised to 230°C (5°C/min) and held for 2 min; the temperature was further raised to 240°C and held for 4 min during the post run.

It is difficult to evaluate the contents of d-Limonene in OPW since it is highly influenced by the extraction conditions and the state of degradation of OPW. Moreover, the evaluation becomes harder still if this measurement must be correlated to the AD of OPW. In fact, essential oil is contained in small sacs in the flavedo (the outer part of the orange peel). The oil is released only when the sacs are broken mechanically (as occurs during the combined extraction of orange juice and EO) or by other methods (e.g. steam distillation as in the Scott method [44]). During the AD of OPW only the EO that is immediately available (e.g. that is already released during juice extraction) has an instant effect following substrate feeding. The remaining amount becomes eventually progressively available during OPW degradation. In this paper we chose to measure only the d-Limonene that was immediately available to the reactor following substrate feeding and for this reason we chose a "mild" extraction. The method used is similar to that previously reported [45].

2.1.3 Semi-continuous reactors

The Organic Loading Rate (OLR) was gradually increased from 0.5 $gVS \cdot L^{-1} \cdot d^{-1}$ in the first week to 1 $gVS \cdot L^{-1} \cdot d^{-1}$ in the second and, finally, 2 $gVS \cdot L^{-1} \cdot d^{-1}$ in the third. During the acclimation period (3 weeks) we used untreated lyophilised OPW to allow a faster adaptation to d-Limonene. From day 21 onwards lyophilised OPW was subjected to the aforementioned NaOH pre-treatment. Feeding was carried out twice a week and hydraulic residence time was set at 13 days. d-Limonene loading in the regime phase (OLR 2 $gVS \cdot L^{-1} \cdot d^{-1}$) was 2.5 $mg \cdot L^{-1} \cdot d^{-1}$. According to previous findings [45] related to tests conducted with hydraulic retention time (HRT) in the same order to that applied here, d-Limonene degradation is highly unlikely; therefore, it accumulates in the reactor leading to a concentration that could be in the order of 33 $mg \cdot L^{-1}$ for the OLR regime (this concentration was obtained in the hypothesis of no

degradation of d-Limonene, multiplying the daily loading for the HRT). This value can be inhibitory for a successful AD [2,6].

Reactor A was terminated after only 50 days of operation due to an excessive reduction in pH while reactor B was terminated after 135 days of operation.

In reactor B OLR was doubled to 4 $gVS \cdot L^{-1} \cdot d^{-1}$ (equivalent to 5.0 $mg \cdot L^{-1} \cdot d^{-1}$ and leading to a concentration of about 65 $mg \cdot L^{-1}$ under the hypothesis drawn of non biodegradability of d-Limonene in the actual experimental conditions) on day 65, but from day 90 it decreased to 3 $gVS \cdot L^{-1} \cdot d^{-1}$ due to the accumulation of VFAs and consequent reduction in pH.

Nutrient solutions were added every two weeks to maintain the desired C/N ratio and, in addition, on days 21 and 28, 3 and 5 g of $NaHCO_3$ was added, respectively, to both reactors to provide an additional buffering capacity due to the excessive pH decrease in reactor A. Furthermore, in reactor B on days 71, 72, 78 and 82 (OLR equals to 4 $gVS \cdot L^{-1} \cdot d^{-1}$) a total of 11.5 mL of NaOH (50% solution, Sigma-Aldrich, St. Louis, MO, USA) was added to increase the pH system.

2.1.4 Digestate characterisation

The pH was measured on the digestate extracted from both reactors twice per week while on average weekly digestate samples VFAs were also evaluated [46,47].

In brief, the method, consisted of a three-point titration of the digestate sample with 0.1 N sulphuric acid at pH 5.0, 4.3 and 4.0. We recorded the volume of the titrant added [46,47]. Before the titration, the digestate sample was centrifuged at 10000 rpm for 10 minutes [47]and then 20 mL of liquid was placed into a beaker where a magnet ensured the constant mixing between the sample and the added acid and an immersed electrode allowed pH measurement .

Total VFAs concentration was calculated as:

$$Total\ VFA\ [mg \cdot L^{-1}] = \left[131340 \cdot \left(V_{pH_{4.0}} - V_{pH_{5.0}}\right) \cdot \frac{N_{H_2SO_4}}{V_{sample}}\right] - \left[3.08 \cdot V_{pH_{4.3}} \cdot \frac{N_{H_2SO_4}}{V_{sample}} \cdot 1000\right] - 10.9$$

$V_{pH_{4.0}}$ Volume in mL of added solution until pH=4.0

$V_{pH_{4.3}}$ Volume in mL of added solution until pH=4.3

$V_{pH_{5.0}}$ Volume in mL of added solution until pH=5.0

V_{sample} Volume in mL of sample (20 mL)

$N_{H_2SO_4}$ Normality of acid solution (0.1)

Only for reactor B, the digestate was collected and filtered once the experiment was terminated. This operation gave the chance to recover GAC, ZVI and iron oxides.

Three different samples were recovered:

1) ZVI/Fe oxides separated by a magnet, rinsed with distilled water and dried (13 g);
2) GAC separated by sieving (mesh $0.85 \cdot 10^{-3} \cdot 0.85 \cdot 10^{-3}$ m) following ZVI/Fe oxides removal (31.2 g);
3) Other components (GAC + ZVI/Fe oxides + remainders of dried digestate) obtained from the rinsing, sieving and drying of the extracted digestate (77.6 g).

GAC and Iron residues were further analysed with an X-ray diffraction (XRD) Scanning Electron Microscope (SEM) to investigate differences from the original structures. Iron residues were also analysed by Temperature Programmed Reduction (TPR) only for ZVI to obtain information concerning their oxidation state [48,49].

2.1.5 16S rRNA amplicon metagenomic analysis

To investigate the bacterial community, liquid samples were taken in triplicates for each reactor after 7, 30, 50, and 135 days of the AD, labelled as time T1, T2, T3 and T4, respectively. Each sample was first vortexed and 15 ml of sample was pre-filtered by a sterile membrane bag to

separate unwanted solid materials (BIOREBA AG, Reinach, BL). The pellet was obtained by centrifugation at 10.000g for 15 min of the filtered phase, and 40 mg were used for total DNA extraction. DNA was extracted with a CTAB modified protocol [50]. Samples were previously omogenised with beads (0.1– 1 mm Ø) at 25Hz for two 1-min periods by using a Mixer Mill homogenizer (Retsch Technology GmbH, Haan, Germany) and a CTAB lysis buffer, excluding mercaptoethanol and adding 1% of fresh PVPP (Sigma-Aldrich, St. Louis, MO, USA). DNA was resuspended in nuclease free water. Concentration and quality of the extracted DNA were evaluated with a Nanodrop ND-1000 spectrophotometer (Nano-drop Technologies, Wilmington, DE, USA). The amplification was carried out by the primer set 799F (AACMGGATTAGATACCCKG) [51] and 1193R (GGAAGGTGGGGATGACGT) [52] targeting the variable regions V5-V7 of the 16S, selected for the ability to avoid the cross-amplification of chloroplasts and mitochondria [53]. The PCR reaction consisted in a total volume of 25 µl containing 12.5 µl of KAPA HiFi Hot Start Ready Mix (KAPA Biosystems, Wilmington, MA, USA), 0.3 µM of each primer, including Illumina adaptors (www.illumina.com), and 50 ng of the DNA template. Reactions were incubated in a Mastercycler gradient thermocycler (Eppendorf, Hamburg, Germany), which was set to have 3 min of denaturation at 95°C, 35 cycles of 30 s at 98°C, of 30 s at 60°C, of 40 s at 72°C, and a final extension of 1 min at 72°C. Amplicons were visualised on gel electrophoresis at 1.2% agarose and sequenced using the MiSeq system according to the protocol provided by the manufacturer for Nextera XT chemistry (Illumina Inc., San Diego, CA, USA). Purifications were carried out by the AgencourtAMPure XP system (Beckman Coulter Genomics, Brea, CA, USA). The final library was normalised and quantified by Qubit (Life Technologies, Carlsbad, CA, USA). The MiSeq reagent kit v3 (Illumina) was used for the long paired-end reads (2 × 300 bp) sequencing run.

The bioinformatics pipeline QIIME v. 1.9.1 was used to process data [54]. PEAR 0.9.10 was utilised to merge paired-end reads, to truncate sequences after two consecutive bases receiving a quality score (Q) < 20 and to delete reads containing one or more ambiguous bases or bases shorter than 280 bp [55]. The *de novo* chimera checking was carried out using the UCHIME algorithm within VSEARCH [56]. Operational taxonomic units (OTUs) were clustered, removing singletons, with a similarity threshold of 97% using the UCLUST-based open-reference OTU-picking workflow while the SILVA database was used to assign taxonomy to representative reads [57,58]. The OTU table was rarefied at an even sequencing depth of 34,600 reads/sample. Non-target sequences were removed by filtering the OTU table. A further quality filtre, based on OTU abundance of 0.005%, was also applied. The alpha-diversity was evaluated using OTU richness, Shannon, and Simpson indexes and the results were statistically compared using a non-parametric two-sample t-test with the False Discovery Rate (FDR) correction method.

Furthermore, to evaluate the diversity of microbial composition between samples, the Bray-Curtis distance matrix was built and statistical significance was tested by the ANOSIM non-parametric test. The Kruskal-Wallis non-parametric test was carried out to identify taxa with significantly different relative abundance (RA). Heatmaps were constructed for each reactor to visualise the results.

3.1 Results and Discussion

3.1.1 Semi-continuous experiments

Both reactors presented a similar behaviour at the very beginning of the experiment (days 0-10). The pH decreased sharply (Figure 1), then it recovered and was equals to about 6.5 on day 28 (about 2 HRT since the beginning of reactor operation). With the proceeding of the days, however, the fate of the two reactors diverged.

In reactor A, the pH decreased rapidly down to 5.2 already after only 20 days of operation (Figure 1), VFAs accumulated (Figure 2), methane yield was about 90 $NmL \cdot gVS^{-1}$ (Figure 3a) and daily methane production declined to about 100 $NmL \cdot d^{-1}$ by the end of the experiment (Figure 3b). In a previous semi-continuous experiment [41] carried out using the same substrate without the benefit of alkali pretreatment, setting a low OLR (1 $gVS \cdot L^{-1} \cdot d^{-1}$) and a long HRT (about 46 days) and where inhibition was not detected, an average methane yield of 380 $NmL \cdot gVSadded^{-1}$ was recorded. Reactor A was terminated after only 50 days of operation due to an excessive reduction in pH. The total CH_4 production cumulated in this reactor was about 4 NL (Figure 4) and the average CH_4 content in biogas at 28-50 days was 48% (Figure 3a). According to these results pre-treatment as well as the GAC addition were not able to guarantee the stable operation of the reactor at a loading equals to 2 $gVS \cdot L^{-1} \cdot d^{-1}$.

In reactor B, the pH was stable, slightly below 7, between days 28 and 65 (about 3 HRT) (Figure 1). The concentration of VFAs tended to decrease (Figure 2), the methane yield steadily increased up to about 200 $NmL \cdot gVS^{-1}$ (Figure 3a) and the methane daily production stabilised around 300 $NmL \cdot d^{-1}$ (Figure 3b). The yield recorded although lower than that reported in [41] is however satisfactory due to the higher loading applied (+100%) and the lower HRT (-70%). On day 50, when the operation of reactor A was terminated, the total CH_4 production cumulated in reactor B (Figure 4) was about 8.5 NL (i.e. 112% higher than reactor A) while the percentage of methane in biogas at 28-50 days was 57% (Figure 3a), about 20% higher than in reactor A. The coupled presence of ZVI and GAC guaranteed the stable and fully satisfactory operation of the reactor at a loading equals to 2 $gVS \cdot L^{-1} \cdot d^{-1}$.

The increase in loading up to 4 $gVS \cdot L^{-1} \cdot d^{-1}$ on day 65 caused the instability of reactor B since the pH dropped down to 5.5 (Figure 1), which was resolved by adding NaOH to stabilise it, as already previously explained. In this time frame VFAs accumulated up to 12 $g \cdot L^{-1}$ (Figure 2). The percentage of methane in biogas dropped down to about 50% (Figure 3a) and methane

production declined (Figure 3b), which were clear signs that methanogenesis was partially inhibited [2].We cannot exclude that the instability of the reactor could have been triggered by the accumulation of d-Limonene that, as already mentioned, could have been in the order of 66 $mg \cdot L^{-1}$. On day 90 and up to the end of the experiment on day 135, the loading was reduced to 3 $gVS \cdot L^{-1} \cdot d^{-1}$, the reactor fully recovered, VFA concentration decreased down to about 4 $g \cdot L^{-1}$ and the methane yield and content in biogas were similar to those observed in the period preceding the loading increase.

The operation of reactor B terminated at day 135, its overall production exceeded 37 NL and the average content of methane in biogas at 50-135 days was 61%, witnessing a possible enhancement of hydrogenotrophs performance. The performance of the reactor was fully satisfactory even if the pH stabilised at a suboptimal value around 6.0. Reactor B was operated at this loading for 45 days (more than 3 HRT), yielding about 200 $NmL \cdot gVS^{-1}$. This value is similar to that obtained by Wikandari and co-authors [11], following chemical leaching of d-Limonene, and by Martins and co-workers [4], following OPW steam distillation. Zema and collaborators [2] found a higher production but with an HRT between 23 and 70 days and an OLR of 0.64 - 1.98 $gVS \cdot L^{-1} \cdot d^{-1}$; they also found that the process was partially inhibited at a loading of about 2 $gVS \cdot L^{-1} \cdot d^{-1}$ and totally inhibited at 2.5 $gVS \cdot L^{-1} \cdot d^{-1}$.

Figure 5 shows the SEM images of GAC (a) and ZVI (b), before use. In the former the porosity is clear while in the latter the structure is compact and solid.

In used GAC (Figure 5c-d), the pores partially filled up, while in used ZVI (Figure 5e-f) the structure changed due to corrosion with possible traces of biological material (g), which were present on the ZVI grains. In fact, Energy-dispersive X-ray (EDX) spectroscopy showed that carbon was the main element present in the little spherical particles present on the ZVI surface. TPR showed that the main oxidation product present in the iron samples was hematite (Fe^{III}); this is a possible indication that microbial corrosion [12] occurred, since the oxidation of Fe^{II}

to Fe^{III} is unlikely under anaerobic conditions [22]; it is, therefore, possible that Fe^{II} was used as an electron donor by bacteria [20].

3.1.2 Metagenomic analysis

Bacterial 16S rRNA sequencing yielded more than 2 million high quality reads. The rarefied OTU table counted 793,800 total reads and 792,435 (99,8%) reads, which were maintained after non-target filtration, were found to belong to the Bacteria kingdom.

Overall, a total of 125 OTUs were assigned to Bacteria lineages identified at different levels, from phylum to genus. For both reactors a decreasing trend was visible in terms of average number of OTUs observed with time. Indeed, 102, 72 and 56 bacterial OTUs were detected at time T1, T2 and T3, respectively, among reactor A samples, while 106, 83, 58 and 49 OTUs were detected at time T1, T2, T3 and T4, respectively, among reactor B samples. However, the two samples t-test analysis, based on the number of OTUs observed, as well as the Shannon and Simpson indexes, did not reveal any significant difference between the samples of both reactors.

At a Phylum level, the bacterial communities appeared to be characterised by the massive presence of Bacteroidetes and Firmicutes, which were globally dominant, with 55.7% and 35%, respectively. Actinobacteria, Proteobacteria and Deinococcus-Thermus were detected with a RA of 1-5%, while the rest of the bacterial communities (the candidate phylum RF3, Synergistetes, Lentisphaerae, Tenericutes, Spirochaetes, Thermotogae, Chlorobi, Chloroflexi and a further candidate phylum) were less represented (less than 1%; refer to Figure 6 for details).

This finding was consistent with other studies in which Bacteroidetes, Firmicutes and Proteobacteria were reported as the most frequent phyla in AD [59–61] and, in particular, Bacteroidetes, which was common in anoxic cellulose treatment [62]. In both reactors the most

abundant phyla were detected at all the time frames considered, but with variable RA; only in reactor B a significant trend where Bacteroidetes rapidly prevailed on the Firmicutes community with time appeared clear, whereas the minor groups appeared to be particularly non-represented by the end of the period of observation, starting from time T3 (Figure 6).

At a genus level, globally both reactors exhibited the major bacterial variability within the 25 most abundant taxa with a RA > 0.5%. Most of the other taxa were detected with a very low RA (Figure 7). The major variability in microbial composition between samples was observed in both reactors at 7 days (time T1), where the two reactors shared a composition that was quite similar, which could be explained by the fact that each reactor incubated the same starting material. The diversity of the bacterial community significantly decreased over time (p-values of 0.004 and 0.001 for reactor A and B, respectively; Figure 7). This trend may be related to the evolution of reactors with more restrictive conditions for the survival of certain groups, indicating a kind of bacterial specialisation to the substrate.

Overall, 103 taxa were found with a RA that was significantly different between samples (p-value ≤ 0.05; Figure 8).

Among them, the analysis focused on those taxa known to be involved in AD systems: *Prevotella*, *Prevotellaceae*, *Oxobacter*, *Clostridiales*, *Proteiniphilum*, *Actinomycineae*, *Ruminococcaceae incertae*, *Lachnospira*, *Lachnospiraceae incertae*, *Clostridium*, *Petrimonas*, *Caldicoprobacter*, *Clostridiales* OPB54, *Fastidiosipila*, *Cystobacteriaceae*, *Lachnospiraceae*, present with a high RA and *Pelotomaculum*, *Lysinibacillus* and *Paenibacillus* present with a low RA (Figure 8).

In both reactors, at 7 days *Clostridiales* and *Proteiniphilum* were dominant, followed by *Prevotella*, *Petrimonas*, *Caldicoprobacter*, *Clostridiales* OPB54 and *Fastidiosipila*. Over time (30, 50 and 135 days) *Prevotella*, and, to a lesser degree, *Prevotellaceae* became dominant,

while *Clostridiales* and *Proteiniphilum* decreased. *Petrimonas*, *Clostridiales* OPB54 and *Fastidiosipila* also decreased over time in both reactors (Figure 8).

It is interesting to highlight the presence of *Pelotomaculum* (0.63% and 0.70% in reactor A and B, respectively) and *Lysinibacillus* in reactor B (Figure 8) at 7 days of the AD. At 30 days (T2), apart from *Prevotella* and *Prevotellaceae*, also *Actinomycineae*, *Lachnospiraceae incertae* and *Clostridium* characterised reactor A, while *Ruminococcaceae* and *Lachnospira* characterised reactor B (Figure 8). At 50 days (T3) *Prevotella* was still consistent in both reactors, but while in reactor A *Oxobacter* dominated with a RA of 42.96%, together with *Actinomycineae*, *Ruminococcaceae* and *Clostridium*, in reactor B *Prevotella* continued to be the most representative (43.78%) followed by *Prevotellaceae*, *Proteiniphilum*, and *Cystobacteriaceae* (Figure 8). *Prevotella*, *Lachnospira* and *Lachnospiraceae incertae* characterised the last stage, T4, which was present only in reactor B (Figure 8).

Some of these bacteria have shown to participate in all phases of AD, such as *Clostridium*, *Lachnospira*, *Prevotella* and almost all acidogenic bacteria also participate in hydrolysis. *Actinomycineae* are known to be hydrolysing bacteria in acidogenesis. *Oxobacter* and *Caldicoprobacter* have been reported to be fermentative, cellulose-metabolising, acetogenic, and iron-reducing bacteria. Also, the genera *Lysinibacillus* and *Paenibacillus* have shown to contribute to iron reduction while the *Cystobacteriaceae*, acetogenic bacteria, are involved in iron oxidation [63]. This is consistent with our findings since the *Cystobacteriaceae* group was present only in the reactor supplied with ZVI that was oxidised during AD.

As results from previous research, *Petrimonas*, *Fastidiosipila*, *Syntrophomonas*, and *Proteiniphilum* are hydrolysing/fermentative bacteria [60,64,65] and play an important role in converting different compounds to acetic acid and CO_2. Species of *Clostridium* and *Lachnospira* have shown pectinase activity under mesophilic conditions [66], producing fatty acids, CO_2, and H_2; *Ruminococcus/Ruminococcaceae* degrade cellulose producing fatty acids

and H_2 [67], while *Prevotella/Prevotellaceae* degrade pectin, producing short chain fatty acids [68,69]. A species of *Pelotomaculum*, a syntrophic propionate-oxidizing bacterium, has been reported to be able to co-aggregate with methanogens and often to interconnect via filament-like structures, enhancing the IET. *Prevotella* has been reported in hydrogen reactors [70] and has the capacity to agglutinate with other microorganisms [71]. It may be possible to hypothesis its involvement in IET. Xu and co-authors [72] reported the genus *Proteiniphilum* as one of the most abundant in carbon-activated reactors. Other authors demonstrated the syntrophic degradation of fatty acids by co-culturing a syntrophic *Proteiniphilum* strain and *Methanobacterium* sp. [73]. Rode and co-workers [74] also reported a case of cross-feeding of a hydrolytic product between *Lachnospira multiparus* and *Eubacterium limosum* also hypothesising IET.

The lack of Archea in this study is most probably due to the choice of the primer. The variable V4 and/or V5 subregion of 16S, which is a popular target for metabarcoding of Bacteria able to reach a high phylogenetic resolution [75], may not offer a suitable resolution for Archea, which have been targeted mostly by the V1-V2 variable subregions [76]. This makes it difficult to design universal primers valid for both lineages. Because of the nature of the starting material (orange waste), primers here were selected, with success, to find a compromise in targeting those 16S regions known to be hypervariable and at the same time excluding the cross-amplification of mitochondria, chloroplasts and the plant genome. This represents a significant challenge in the selection of appropriate primer pairs to study plant-microbe interactions starting from plant tissue [52,53,77,78]. Moreover, the primer choice has been recognised as a fundamental issue in amplicon metagenomic analysis, based on both 16S rRNA and ITS regions. Indeed, several metabarcoding studies demonstrated that, because of the structure of these genomic regions, the primer choice can greatly affect the results, in terms of taxa composition, detecting preferentially certain taxa [79,80], but also limiting the microbial

lineages coverage, recovering from 50% to 80% of the total variability even when two different primer sets are combined [81]. However, the lack of Archea was not predictable as the aforementioned studies also showed that the actual performance of the primers did not always conform to the *in silico* evaluation, highlighting the need for experimental assays.

4.1 Conclusions

The AD of OPW would be greatly beneficial for its management but sound solutions have yet to be found. According to the results of this paper alkaline pre-treatment as well as the addition of GAC were insufficient to guarantee the stable operation of the reactor at an OLR of 2 $gVS \cdot L^{-1} \cdot d^{-1}$; in fact, the operation of Reactor A was terminated after only 50 days due to the low pH value. The alkaline pre-treatment and addition of GAC, as well as ZVI, guaranteed better results. In these conditions, we observed a stable operation of the reactor at an OLR up to 3 $gVS \cdot L^{-1} \cdot d^{-1}$, a sustained methane production (about 200 $NmL \cdot gVS^{-1}$) and a suitable percentage of methane in biogas (61%) produced. The investigation of the bacterial community, by high-throughput sequencing, also increased our knowledge concerning their involvement in AD in the presence of ZVI, including its biotic oxidation. In light of the results obtained it is likely that direct interspecies electron transfer played a role in the reactor supplemented with ZVI.

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