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Abstract

The valorisation of olive oil mill wastewater (OMW) through anaerobic digestion requires identifying the concentration of polyphenols (PP) that causes failure of the process of digestion. In addition, the advantages of the possible microbial adaptation, in terms of increased methane production, to significant concentrations of PP as well as the kinetics of OMW anaerobic degradation requires evaluation. To fill these knowledge gaps, anaerobic digestion batch tests were carried out on three blends of OMW and inoculum (digestate from a biogas plant fed with agro-wastes) at a PP concentration of 0.5, 1.0 and 2.0 g l^{-1} in mesophilic conditions. Total inhibition of anaerobic digestion was found at a PP concentration of 2.0 g l^{-1} (non-adapted inoculum group). A positive effect of the adaptation to the substrate was, instead, observed for the blends with adapted inoculum at a PP concentration of 1.0 and 2.0 g l^{-1}. Methane yields increased by 70% (PP = 1.0 g l^{-1}) and 300% (2.0 g l^{-1}) in the group with adapted inoculum compared to the group with non-adapted inoculum. The results suggest that OMW should not be subject to anaerobic digestion at high PP concentrations (i.e. higher than about 1 g l^{-1}) due to the microbial inhibition detected. Moreover, given the benefits of the adaptation of the microbial population that was more evident at the highest PP concentration tested, it is advisable to allow the progressive adaptation of the digestion to OMW feeding. Thanks to the increased methane yield, because of the improved microbial tolerance to inhibiting compounds, the anaerobic digestion of OMW could be a viable and environmentally sound solution for the treatment of agro-industrial wastewater.

Keywords: anaerobic digestion; oil residues; inhibiting compound; methane yield; microbial adaptation; biogas production.

Nomenclature

OMW	Olive Mill Wastewater
PP	Polyphenols
TS	Total Solids
TVS	Total Volatile Solids
DM	Dry Matter
COD	Chemical Oxigen Demand
BMP1	Series of tests carried out with raw digestate as inoculum
BMP2	Series of tests carried out with adapted digestate and BMP1 as inoculum
T_{50}	Time needed to get 50% of the maximum methane yield
T_{90}	Time needed to get 90% of the maximum methane yield
B	methane production of the 1st-order kinetics model
B_0, M, y	cumulative methane production of the 1st-order kinetics, Gompertz or logistic models
k	constant of the 1st-order kinetics or logistic models
t	time for digestion of the 1st-order kinetics, Gompertz or logistic models
P	methane potential of Gompertz model
R_m	maximum methane production rate of Gompertz model
λ	lag phase period of Gompertz model
a, b	constants of logistic model
WW	Winery Waste
PW	Pig Waste
OMSW	Olive Mill Solid Waste
AW	Abattoir Wastewater
LPM	Liquid Poultry Manure

1. Introduction

The olive oil industry, which is one of the most traditional agricultural industries in the Mediterranean region, generates large amounts of residues: a very wet, plastic olive cake, the so-called "olive pomace", and a liquid stream, called "olive oil mill wastewater" (OMW), which is produced by the wastewater generated during the different stages of the process in olive oil production and by the water used for cleaning purposes (Moreno et al., 2017). On a broad scale,

olive processing produces 50% wastewater, 30% solid residues and 20% olive oil (Komnitsas and Zaharaki, 2012). The major environmental problems associated with olive oil extraction mills are related to both the large volumes of water required and the ineffective management of OMW and olive pomace (Dourou et al., 2016). OMW composition presents a large diversity depending on several parameters, such as the variety of olives and their maturity, the region of origin, and especially the technology used for oil extraction (Roig et al., 2006). However, OMW is always characterised by high concentrations of several organic compounds (e.g. organic acids, tannins and phenolic compounds), which make it difficult to treat due to its resistance to biodegradation (Turano et al., 2002) causing serious environmental concerns when its management is not environmentally sound. For instance, OMW discharged into water courses can lead to water body deterioration with significant damages to the aquatic life (Karaouzas et al., 2011), while pollution of groundwater, soil contamination, production of unpleasant smells, as well as the toxicity of vegetation are also possible in case of uncontrolled disposal and/or insufficient treatment of OMW (Aggelis et al., 2003). Therefore, due to its polluting power and to the increasing severity of the applicable legislation (Gòmez et al., 2010), the disposal of residues from oil extraction has become a major concern for olive oil producers. The need to apply suitable management practices, which are able to combine environmental and economic sustainability for olive oil facilities, is now clear.

Currently, the methods applied for OMW treatment are either physico-chemical (e.g. simple evaporation, reverse osmosis, ultrafiltration, coagulation, oxidation, thermal drying and advanced oxidation processes) or biological (aerobic treatment, composting, vermicomposting together with other agro-industrial residues), but the majority of these techniques are complex and expensive and the results obtained are often poor (Gomez et al., 2010; Komnitsas and Zaharaki, 2012; Dourou et al., 2016).

Anaerobic digestion has been proposed as a promising technology for the valorisation of olive oil residues through biofuel production (i.e. biogas) (Ponsà et al., 2011; Sheng et al., 2013), since this process can be carried out by applying relatively inexpensive and simple reactor designs and operating procedures (Tekin and Dalgıc, 2000). However, researchers must overcome many issues (such as low pH and nitrogen content, alkalinity, presence of inhibiting compounds) for this technology to be applied to OMW (Orive et al., 2016). Most researches have attributed the related problems observed during anaerobic digestion to the presence of polyphenols (PP). However, these experiments were often carried out using synthetic wastewater containing cellulose or acetate as main substrate (Madiguo et al., 2016; Wang et al., 1991; Chapleur et al., 2016) or even PP as substrate (Field and Lettinga, 1987; Fedorak and Hrudey, 1984). High concentrations of PP in the anaerobic digestion of OMW lead to very low biogas and methane production rates and consequent

reduced treatment efficiency. Moreover, the reduced energy yield reduces the economical convenience of the anaerobic digestion of OMW.

In general, typical PP concentrations of OMW are in the range of 0.5 - 24 g l^{-1} (Borja et al., 2006; Gonzalez-Lopez et al., 1994), but severe methane yield reductions have been noticed already at PP concentrations of about 0.5 - 2 g l^{-1} (Fedorak and Hrudey, 1984; Borja et al., 1996). This means that raw OMW should be either co-digested with other organic substrates or fed with limited loads to the digester.

The scientific literature reports of many experimental tests concerning the anaerobic digestion of olive residues, often in co-digestion with other substrates. For instance, as co-substrates, Fontoulakis et al. (2008) used winery waste, Athanasoulia et al. (2012) activated sludge waste and Dareioti et al. (2009) cheese whey and liquid cow manure. Pre-treatments for the removal of PP compounds were also explored focusing on the possibility of increasing methane yield (e.g. biological treatment with yeast or fungi, Gharsallah et al., 1999; Martinez-Garcia et al., 2009; Borja et al., 1995; aerobic treatment, González-González and Cuadros, 2015; chemical treatment, Siciliano et al., 2016).

Co-digestion is the most common practice to reduce the negative effects associated with the inhibiting compounds, since pre-treatments are often expensive and not sustainable by the smallest olive oil mills. However, co-digestion requires knowing which is the OMW maximum concentration tolerated by the anaerobic process to obtain an acceptable energy yield.

Enhancing the tolerance of the anaerobic processes towards PP through microbial adaptation may be a simple method to increase methane yield during OMW digestion. To explore this possibility, it is firstly necessary to investigate which is the PP limit concentration to avoid the inhibition on the methanogenic activity. Some studies are available on the effects of PP on OMW anaerobic digestion, but often data are scattered due to the different conditions adopted for the process (e.g. for co-digestion: hydraulic retention time, organic loading rate, Fountoulakis et al., 2008; Battista et al., 2013; Dareioti, 2009, 2010) and for the different inocula used (e.g. Gonzàles-Gonzàles and Cuadros, 2015, Fezzani and Chiekh, 2008; Kougias et al., 2010, 2014). The advantages of microbial adaptation to PP must be further evaluated by a quantitative approach; in other words, the increase of methane yield due to the increased tolerance of OMW to PP has to be measured by suitable Biochemical Methane Potential (BMP) tests, in order to evaluate the maximum methane yield that could be obtained by using an acclimated inoculum. The increase of methane yield by microbial adaptation may increase the appeal of anaerobic digestion as one of the most profitable methods for OMW valorisation in the olive oil production sector.

Unfortunately, the number of investigations on microbial population adaptation to PP contained in OMW is limited and they are mainly carried out as OMW co-digestion (Kougias et al., 2010, 2014; Fezzani and Cheikh, 2008, Gannoun et al., 2007); however, a quantitative evaluation of the potential increase in methane yield due to microbial adaptation has not to the best of our knowledge, been performed. Furthermore, to evaluate the accuracy of the literature models (e.g. first-order kinetics, Gompertz and logistic models) in simulating the methane production rates observed in the BMP test (Durruti et al., 2012) could also turn useful; again no experiments have been conducted on this topic, to the best of our knowledge.

In order to fill these knowledge gaps, this paper evaluates the PP concentration in the anaerobic reactor that induces inhibition of OMW anaerobic digestion, by comparing non-adapted and adapted inocula. More specifically, through batch tests under mesophilic conditions at three PP concentrations we: (i) evaluated the concentration of PP that leads to the inhibition of OMW anaerobic digestion; (ii) measured the increase in methane yield due to the microbial adaptation of the inoculum to PP, compared to the non-adapted inoculum, and (iii) tested the accuracy of the literature models (first-order kinetics, Gompertz and logistic models) in simulating the methane yields measured in our tests. More specifically, modelling is an essential tool for a synthetic analysis of the outcome of the experimental activity and is also useful to set the operational parameters during the scaling up of the process to pilot or full-scale plants dimensions.

2. Materials and methods

2.1 Biochemical Methane Potential (BMP) tests

Two series of anaerobic batch tests (designated BMP1 and BMP2) were carried out. The first series (BMP1) consisted of three tests with digestate (taken from an external plant and used as *inoculum*) and OMW blended at different PP concentrations (0.5, 1.0 and 2.0 g l^{-1}, indicated below as $PP_{0.5}$, $PP_{1.0}$ e $PP_{2.0}$, respectively), each one in triplicate. This BMP1 series was aimed at evaluating the biogas/methane yield reduction at increasing PP concentrations or, in other words, the PP concentration that inhibited the anaerobic process.

Subsequently, the digestate produced during BMP1 tests (adapted digestate) was mixed and used as inoculum of the BMP2 test at the same PP concentration. The objective was to evaluate whether an inoculum, already subject to anaerobic digestion in the presence of a significant PP concentration and thus characterised by an adapted microbial population, played a positive effect not only on biogas production, but also on the capability of bacteria to tolerate a higher PP concentration when compared to a non-adapted inoculum. BMP2 tests were carried out at PP concentrations of 1.0 and 2.0 g l^{-1}, respectively (two reactors for each PP concentration). For each of the two series of tests, a

fourth test (control) was carried out using only inoculum, in order to evaluate endogenous biogas yields. A schematic representation of the BMP design is shown in Fig. 1.

2.2 Analytical methods

The digestate used in the BMP1 tests (raw digestate) was taken from a commercial biogas plant, fed mainly with manure and other agro-waste.

OMW was extracted by a 3-phase olive oil mill. However, raw OMW showed a low PP concentration (1.05 g l^{-1}), presumably under the expected inhibition limit. Therefore, OMW was concentrated in an oven at 60°C (in any case, below the evaporation temperature of the PP) until the PP concentration in the final volume was about 4-fold the initial concentration (Table 1).

The raw digestate and OMW were previously characterised, by measuring the Total Solids (TS, by oven drying the wet biomass at 70°C until reaching weight stabilisation), Total Volatile Solids (TVS, by calcination of the dry matter), pH (a portable pH-meter, XS Instruments) and C/N ratio (Leco series 628, United States). COD and PP concentrations in the OMW were measured. COD was determined on diluted (1:10 v/v) effluents by cuvette cap tests (WTW, code 1.14555, photometer WTW, PhotoLab S12). The measurements were carried out in triplicate. PP concentration was measured by the Folin-Ciocalteu colorimetric method (Folin and Ciocalteu, 1927) and expressed as g l^{-1} of gallic acid, measured by a spectrophotometer (PerkinElmer, Lambda 35 UV-VIS). Table 1 reports the main physico-chemical parameters of the substrates (OMW and digestate) used in the two series of tests.

Table 2 reports the characteristics of the four blends subject to the BMP1 and BMP2 tests.

Table 1

Table 2

All batches of both series were kept for 30 d under mesophilic conditions (35°C). Before the beginning of the test, sodium bicarbonate was added to increase the buffering capacity. Each test was carried out in triplicate (BMP1) or duplicate (BMP2, due to the lack of sufficient amount of acclimated inoculum) in a hermetic bottle, which was continuously stirred by a magnetic bar.

Throughout the experiment, biogas and methane production were measured three times a week. The volume of biogas produced was measured according to Calabrò et al. (2016, 2018); the methane content in the biogas was estimated by the fluid displacement method using a 3M NaOH solution (Calabrò et al., 2016, 2018). The average methane yields (evaluated at standard pressure and

temperature conditions) of the control tests were subtracted from the corresponding values of the other tests, in order to estimate the net specific methane yield.

The time needed to get 50% and 90%, respectively, of the maximum methane yield (hereinafter indicated as "T_{50}" and "T_{90}") was also evaluated.

2.3 Experimental design and statistical analysis

The design of the experiment followed a 14 × 3 (BMP1) or 14 × 2 (BMP2) factorial layout, with 14 sampling dates of daily specific methane yields and 3 (BMP1, each batch with 3 replicates) or 2 (BMP2, each batch with 2 replicates) PP concentrations (at $p < 0.01$). This factorial layout, which was analysed by a two-way ANOVA using the PAST software (Hammer et al., 2001), was aimed at evaluating whether there was an interaction between time and PP concentrations.

Subsequently, based on the outcome of the two-way ANOVA, using single factor analysis of variance (ANOVA) along with Tukey's test (designed for the pairwise comparisons), statistical analysis of cumulated specific methane yield from batch anaerobic digestion was performed. The significance threshold was set at $p < 0.05$. More specifically, pairwise comparisons were applied to: (i) different PP concentrations (separately for the BMP1 and BMP2 batches); (ii) BMP1 and BMP2 batches at the same PP concentration (separately for PP = 1.0 and 2.0 g l^{-1}). Moreover, pairwise comparisons by Tukey's test were also used to evaluate the statistical significance in variability of daily specific methane yields (i.e. the difference in slopes of the cumulated production rates) throughout the anaerobic digestion cycle.

The ANOVA analysis of variance assumes that the residuals are normally distributed. Thus, data from batch experiments were also tested for the assumption of normality by using the Aderson-Darling methodology, which is based on the function of empirical distribution. This statistical analysis was performed using Minitab Statistical Software release 17 (Minitab, Inc., State College, Pennsylvania, USA).

2.4 Analysis of degradation kinetics

The kinetics and the experimental methane yields were finally simulated by first-order kinetics, modified Gompertz and logistic models (Donoso-Bravo et al. 2010, Ghatak and Mahanta, 2014):

1^{st}-order kinetics:

$$B = B_0 \cdot \left[1 - \exp^{-kt}\right] \quad (1)$$

where B is methane production, l g^{-1} [TVS]; B_0 is cumulative methane production (maximum methane yield), l g^{-1} [TVS]; k is first order kinetic constant, d^{-1}; and t is time for digestion, d.

237

238 *Gompertz (modified equation):*

239 $$M = P \cdot \exp^{-\exp^{\frac{R_m \cdot e}{P}(\lambda - t)+1}} \quad (2)$$

240 Where M is cumulative methane production, l g^{-1} [TVS]; P is methane potential , l g^{-1} [TVS]; R_m is
241 maximum methane production rate, l g^{-1} [TVS] d^{-1} ; λ is lag phase period or the minimum time
242 required to produce biogas, d; and t is time for digestion, d.

243

244 *Logistic model:*

245 $$y = B\frac{a}{1 + b \cdot \exp^{-kt}} \quad (3)$$

246 Where *y* is cumulative methane production, l g^{-1} [TVS]; t is time for digestion, d; *k* is kinetic rate
247 constant, d^{-1}; and *a*, *b* are constants.

248

249 **3. Results**

250 *3.1. Batch tests with non-adapted inoculum (BMP1)*

251 The maximum cumulative value (0.419 Nl g^{-1} [TVS]) of the net specific methane yield was
252 observed in the $PP_{0.5}$ batch. With 2-fold and 4-fold PP concentrations (the $PP_{1.0}$ and $PP_{2.0}$ blends)
253 methane yields were, respectively, 43% (0.244 Nl g^{-1} [TVS]) and 89% (0.045 Nl g^{-1} [TVS]) lower
254 (Fig. 2b and Table 3).

255

256 Figure 2

257

258 Table 3

259

260 The batches with the highest PP concentration reached 50% of the maximum methane yield just
261 after 5.6 d, thus showing an early blockage of methanogenic activity; the other blends showed T_{50}
262 of 11.1 ($PP_{0.5}$) and 14.6 ($PP_{1.0}$) days, while the methane production was practically completed after
263 20-25 days (Table 3). The methane production shown by the $PP_{1.0}$ batches increased almost
264 constantly throughout the experiment.

265 The cumulative production of biogas was similar to that of methane; the biogas yield was 0.614,
266 0.385 and 0.116 Nl g^{-1} [TVS]for the $PP_{0.5}$, $PP_{1.0}$ and $PP_{2.0}$ blends, respectively (Fig. 2a). The highest
267 biogas content, which was averaged during the overall process, was detected for the $PP_{0.5}$ (58%)

and $PP_{1.0}$ (47%) blends, while, in contrast to the other blends, a noticeable reduction of the biogas percentage was observed in $PP_{2.0}$ (28%).

For all the batches pH values were always in the optimal range (7-8) required for a balanced anaerobic digestion (Gonzàles-Gonzàles and Cuadros, 2015; Fezzani and Cheikh, 2008; Martinez-Garcia et al., 2009). The lowest final pH was measured for $PP_{2.0}$ (Table 4), presumably linked to a higher concentration of acids (not consumed by methanogenic bacteria).

The maximum correlation level with our experimental data was observed with the Gompertz model ($r^2 = 0.96$, $PP_{2.0}$ batch, with a maximum value of 0.99 for $PP_{0.5}$ and $PP_{1.0}$), when simulating the net methane specific yields by the literature models.

Table 5 shows the parameters obtained with the modified Gompertz equation determined by the "Solver" function of Excel® (Microsoft® Office®) application, minimising the differences between experimental and modelled cumulative methane production (least squares method). The parameters of the model confirmed the adverse effect of increasing PP concentration both in terms of final yield and maximum production rate.

Table 4

Table 5

3.2. Batch tests with adapted inoculum (BMP2)

In the BMP2 tests (adapted inoculum), the cumulated net specific methane yield, measured for the two blends, $PP_{1.0}$ and $PP_{2.0}$, was 0.419 and 0.170 Nl g^{-1} [TVS], respectively (Table 3). T_{50} and T_{90} were higher than in the BMP1 tests (Table 3).

The biogas yield was on average 0.602 and 0.286 Nl g^{-1} [TVS] for the $PP_{1.0}$ and $PP_{2.0}$ blends, respectively (Fig. 3). The test also showed a high content in methane (65%, $PP_{1.0}$, and 58%, $PP_{2.0}$; Fig. 2).

As found for the BMP1 tests, the best simulation of our experimental data was given by the Gompertz model for both the $PP_{1.0}$ e $PP_{2.0}$ batches ($r^2 = 0.99$ in both cases; Table 5). Modelling confirmed the positive effect of inoculum adaptation; in particular, the maximum production rate for BMP2 at a PP concentration equal to 1.0 g $l^{-1}$was very close to that of BMP1 at a PP concentration equal to 0.5 g l^{-1}. From a practical point of view, this implies that to roughly obtain 0.25 Nl g^{-1} [TVS]of methane in a reactor containing 1.0 g l^{-1} of PP 31 d are needed with a non-adapted inoculum while only 19 d are sufficient with an adapted one.

Figure 3

4. Discussion

The analysis of the specific methane yields measured for the two BMP series highlighted noticeable differences among blends at different PP concentrations. For both the BMP1 and BMP2 series, the two-way ANOVA highlighted that the factors "sampling time" and "PP concentration" were statistically significant, when considered separately, but that their reciprocal interaction was not significant (for $p < 0.01$).

In the BMP1 test, the statistical analysis (one-way ANOVA together with Tukey's test for pairwise comparisons) showed that only the difference between $PP_{0.5}$ and $PP_{2.0}$ was statistically significant ($p < 0.05$). The $PP_{0.5}$ batch produced methane at a significantly higher rate ($p < 0.05$) until the 20th day compared to the same production measured at a later stage (days 21-30). Moreover, the methane production rate of the $PP_{0.5}$ batch was significantly ($p < 0.05$) higher compared to $PP_{1.0}$ in the first 20 d, while the difference in production rate between the two batches became not significant ($p < 0.05$; Figure 2b) during the course of the days (days 21-30).

In the BMP2 test, the cumulated net specific methane yield, measured for the two blends, $PP_{1.0}$ and $PP_{2.0}$, was significantly different between the two batches ($p < 0.05$; Fig. 3). A slow increase in net specific methane yield was detected for both batches during the first ten days of the experiment; this production increased at a higher rate during the course of the following ten days, although not significantly compared to the previous period. The difference in methane production rates of the $PP_{1.0}$ e $PP_{2.0}$ batches was significant ($p < 0.05$) only at the lowest PP concentration (Fig. 4). This outcome suggests a possible adaptation of the microbial population to the inhibiting compounds in the batch.

Figure 4 compares the net specific methane yields of the BMP1 (raw inoculum) and BMP2 (inoculum adapted to PP) tests. The significant increase of the specific methane yield detected in the tests may be attributable to the microbial tolerance to high PP concentrations developed in the previous digestion cycle. More specifically, the net specific methane yield (0.419 Nl g^{-1} [TVS]) of the $PP_{1.0}$ blend in the BMP2 test was higher by 72% compared to the same (0.244 NL g^{-1} [TVS]) in the BMP1 test (Table 3) although results were not significant. In contrast, the net specific methane yield (0.17 Nl g^{-1} [TVS]) of the $PP_{2.0}$ blend in the BMP2 test was even 4-fold to that observed (0.045 Nl g^{-1} [TVS]) in the BMP1 test and results were significant ($p < 0.05$; Table 3). The average methane percentage also increased by using the adapted inoculum (from 47 to 65%, $PP_{1.0}$, and from 28 to 58%, $PP_{2.0}$), which seems to confirm the positive role of the microbial adaptation on the methanogenic process.

Figure 4

The analysis of the process parameters modelled by the literature models (Table 5) highlighted that the adapted inoculum increased both the maximum methane production rate (from 0.010 to 0.018 Nl g^{-1} [TVS] d^{-1} for the $PP_{1.0}$ batches and from 0.001 to 0.007 Nl g^{-1} [TVS] d^{-1} for the $PP_{2.0}$ batches) and the potential in specific production (from 0.317 to 0.615 Nl g^{-1} [TVS] for the $PP_{1.0}$ batches and from 0.235 to 0.478 Nl g^{-1} [TVS] for the $PP_{2.0}$ batches). During the BMP1 test the maximum tolerated PP concentration that did not lead to process inhibition was about 0.5 g l^{-1}, while in the BMP2 test this concentration increased to about 1 g l^{-1}. Lag periods were higher in the BMP2 test (4.61 and 7.73 d, for the $PP_{1.0}$ and $PP_{2.0}$ batch, respectively) than in the BMP1 test (Table 5), probably due to non-degraded PP in the digestate derived from BMP1; however, methane production quickly recovered after the adaptation phase for both blends.

These results suggest that the adaptation of the inoculum significantly increases the tolerance to the inhibition effects played by phenolic compounds. An incremental feeding of OMW in anaerobic digestion plants, starting from very limited amounts, could enhance microbial adaptation. This could be a possible and cheap solution, compared to the more expensive pre-treatments, to significantly increase the methane yields of OMW with a non-negligible content of PP. This option, which requires the use of large tanks for OMW storage, could optimise the PP concentration in the reactor and keep the microbial population adapted to these substrates. Furthermore, OMW storage in the tanks would allow its availability as substrate for anaerobic digestion throughout the year, thus avoiding the limits linked to its seasonal production (concentrated from October to February).

Some interesting considerations were drawn when we compared the results of our batch tests with other experiments reported in the literature regarding anaerobic digestion tests conducted with OMW blends and at mesophilic conditions (Table 6):

- the highest methane yields of our study (the $PP_{0.5}$ and $PP_{1.0}$ blends) without microbial adaptation (205 and 353 Nml g^{-1} [COD_{added}], respectively) were comparable to those observed by Fontoulakis et al. (2008) (214 Nml g^{-1} [COD_{added}]);
- conversely, our $PP_{2.0}$ blend (in which inhibition was evident) showed the lowest methane yield among all the studies reported;
- with microbial adaptation, only the $PP_{1.0}$ blend showed a methane yield (275 Nml g^{-1} [COD_{added}]) similar to the findings of Kougias et al. (2010, 2014) (277 Nml g^{-1} [$COD_{removal}$] and 480 Nml g^{-1}[TVS]) in which the experiment evaluated the co-digestion of OMW and swine manure;

- the PP concentration that showed severe inhibition (2.0 g l^{-1}) was similar to that determined by Gannoun et al. (2007).

Table 6

As previously mentioned, the majority of studies concerning PP inhibitory concentrations were carried out on synthetic wastewater containing specific phenolic compounds. In other experiences, mesophilic anaerobic digestion of OMW was carried out in co-digestion with other organic wastes, or in semi-continuous mode, and without an acclimated inoculum (Azbar et al., 2009; Fountoulakis et al., 2008; Fezzani and Cheikh, 2010; Athanasoulia et al., 2012; Dareioti and Kornaros, 2014). The BMP experiments carried out on an acclimated inoculum were also performed in co-digestion; moreover, different methods were used to acclimate the inoculum. Kougias et al. (2010), who used an inoculum of digested sludge obtained from an anaerobic reactor fed with OMW and pig waste (PW) at the optimal ratio of 40:60 (v/v), measured a maximum methane yield of 480 Nml g^{-1} [TVS] (corresponding to 297 Nml g^{-1} [COD_{added}]). The same authors found a methane yield of 370 Nml g^{-1} [TVS] (corresponding to 277 Nml g^{-1} [$COD_{removed}$]) at the same optimal ratio (Kougias et al., 2014). Khoufi and co-workers (2015) used the anaerobic stock culture from a semi-pilot anaerobic bioreactor fed with OMW as inoculum; the optimal ratio for the co-digestion of OMW and liquid poultry manure (LPM) in batch reactors was 70:30 (v/v), corresponding to a PP concentration of 6.83 g l^{-1}, although the methane yield was only 55.1 Nml g-1[TS]. Fezzani and Cheikh (2008) studied the efficiency of the co-digestion of OMW and olive mill solid waste (OMSW); digestion of only OMW produced 11.17 l of biogas per l of digester (PP concentration of 6 g l^{-1}) and this biogas production increased to 30.5 l l^{-1}[digester] by co-digestion of OMW with OMSW (PP concentration of the mixture equal to 3 g l^{-1}). Finally, Gannoun and collaborators (2007) carried out a toxicity test in order to evaluate the inhibitory PP concentration in different mixtures of OMW and abattoir wastewater (AW) at the optimal ratio OMW:AW of 40:60 (v/v) and PP concentration of 2.04 g l^{-1}: the biogas yield after 10 days was 0.189 l per g [$COD_{removed}$]; the inoculum used was taken from an active mesophilic digester treating OMW.

In all the aforementioned cases, the tests were aimed at measuring the optimal ratio between OMW and the other co-substrates; none of these studies identified the inhibitory PP concentration or evaluated the beneficial effect of the acclimated inoculum on the increase of the bio-methane production. Our BMP tests, instead, were successful in identifying the concentration of polyphenols inhibiting the anaerobic digestion of OMW (close to 0.5 g l^{-1} without microbial adaptation) and in quantifying the advantages of the microbial population adapted to phenols (by measuring the

403 increase of methane production - from about 70% to 280% - compared to the non-adapted
404 substrate).
405
406 **5. Conclusions**
407 The batch tests revealed that:
408 - A PP concentration of 2.0 g l^{-1} induced an early blockage of methanogenic activity, while
409 the lowest PP (PP 0.5 g l^{-1}) gave the highest methane yield (0.419 Nl g^{-}1 [TVS]);
410 - When the digestate of the first tests was used as inoculum in the second series, the net
411 specific methane yield of the blend with 1.0 g l^{-1} PP was 72% higher compared to the
412 BMP1 test, while the blend with 2.0 g l^{-1} PP showed a methane yield that was 4-fold higher;
413 - The change described above was attributed to the adaptation of the bacterial population to
414 PP, suggesting that OMW should be fed to anaerobic digestion by initially keeping PP
415 concentrations always lower than 1.0 g l^{-1} and by then gradually increasing PP
416 concentrations in order to allow the full exploitation of the mechanisms of adaptation;
417 - The net methane specific yields were well simulated by using the modified Gompertz
418 equation;
419 - Modelling allowed to evaluate potential practical implications of inoculum adaptation to PP;
420 - It is feasible to consolidate the idea that a potentially pollutant organic waste (i.e. the olive
421 processing residues) can be turned into a profitable source by anaerobic digestion
422 technology, thus enhancing a sustainable and cleaner production of olive oil.
423
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430
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553 **TABLES**

554

555 Table 1 - Main physico-chemical parameters of the substrates (OMW and digestate) used in the two
556 series of anaerobic digestion tests (BMP1 and BMP2).

557

Parameter	Test					
	BMP1			BMP2		
	Inoculum	*Raw* OMW[1]	*Concentrated* OMW[1]	*Inoculum*	*Raw* OMW[1]	*Concentrated* OMW[1]
TS[2,*] [%]	5.9±0.05	3.4±0.07	12.7±0.1	7.30±0.05	3.4±0.04	10.1±0.1
TVS[3,*] [% on DM[4]]	75.2±0.65	70.4±1.95	72.4±0.5	62.5±1.8	84.3±0.9	70.3±2.2
COD[**] [g l^{-1}]	-	25.6±0.3	109.3±0.42	-	47.1±1.4	107.7±2.72
PP[5,**] [g l^{-1}]	-	1.1±0.12	4.4±0.03	-	1.2±0.08	4.2±0.08
C/N[*]	13.8±0.2	37.2±0.1	40.9±0.15	16.4±0.14	27.1±0.2	29.2±0.3
pH[*]	8.4±0.01	4.2±0.02	4.5±0.01	8.4±0.02	4.3±0.01	4.5±0.02

558 Notes: [1]OMW = Olive Oil Mill Wastewater; [2]TS = Total Solids; [3]TVS = Total Volatile Solids; [4]DM = Dry Matter; [5]PP
559 = Polyphenols; * n = 2 replicates; ** n = 3 replicates.

560

561

Table 2 - Main physico-chemical parameters of the blends subject to the BMP tests.

	Parameter	**Test**						
		BMP1				**BMP2**		
		Inoculum	$PP_{0.5}$	$PP_{1.0}$	$PP_{2.0}$	Inoculum	$PP_{1.0}$	$PP_{2.0}$
Inoculum	Volume [ml]	200						
	TS,[2,*] [g]	11.8±0.08				14.6±0.08		
	TVS[3,*] [g]	8.9±0.06				9.2±0.22		
Concentr. OMW[1]	Volume [ml]	-	26	60	170	-	63	183
	TS[2,*] [g]		3.4±0.02	7.6±0.05	21.6±0.14		6.3±0.05	18.4±0.15
	TVS[3,*] [g]		2.4±0.02	5.5±0.04	15.6±0.13		4.5±0.06	12.9±0.33
	COD[**] [g]		2.8±0.01	6.6±0.02	18.6±0.06		6.7±0.001	19.7±0.0004
	PP[4,**] [g l^{-1}]		0.5±0.003	1.0±0.001	2.0±0.001		1.0±0.009	2.0±0.032

Notes: [1]OMW = Olive Oil Mill Wastewater; [2]TS = Total Solids; [3]TVS = Total Volatile Solids; [4]PP = Polyphenols; COD = Chemical Oxygen Demand; * n = 2 replicates; ** n = 3 replicates.

570 Table 3 – The net specific methane yield (in Nl g^{-1} [TVS]) after 30 d and number of days for 50%
571 (T_{50}) and 90% (T_{90}) of maximum yield.
572

Test	Net specific methane yield	Time	
	Maximum	**T_{50}**	**T_{90}**
BMP1			
$PP_{0.5}$*	0.419	11.1	20.8
$PP_{1.0}$*	0.244	14.6	24.4
$PP_{2.0}$*	0.045	5.6	27.3
BMP2			
$PP_{1.0}$**	0.419	16.4	26.4
$PP_{2.0}$**	0.170	18.0	27.8

573 Notes: PP = Polypenols; T_{50}, T_{90}= Time needed to get 50% and 90% of the maximum methane yield; * n = 2 replicates;
574 ** n = 3 replicates.

576 Table 4 – Mean values of initial and final pH in the BMP1 and BMP2 tests (mean of 2 replicates).

577

pH	**Inoculum**	**$PP_{0.5}$**	**$PP_{1.0}$**	**$PP_{2.0}$**
BMP1				
Initial	8.52±0.01	7.71±0.02	7.28±0.04	7.03±0.02
Final	8.05±0.02	7.89±0.03	7.92±0.05	6.72±0.02
BMP2				
Initial	8.46±0.02	-	7.19±0.04	7.05±0.07
Final	7.92±0.02	-	7.97±0.02	7.80±0.14

578 Note: PP = Polyphenols.

580 Table 5 – The parameters obtained by the modified Gompertz model.

581

Test	P [l g^{-1}[TVS]][1]	R_m [l g^{-1} [TVS] d^{-1}][1]	λ [d]	R-square [-]
BMP1				
$PP_{0.5}$	0.468	0.022	1.06	0.999
$PP_{1.0}$	0.317	0.010	2.73	0.997
$PP_{2.0}$	0.235	0.001	0.00	0.963
BMP2				
$PP_{1.0}$	0.615	0.018	4.61	0.994
$PP_{2.0}$	0.478	0.007	7.73	0.996

582 Notes: [1]TVS = Total Volatile Solids; P = methane potential; R_m = maximum methane production rate; λ = lag phase
583 period.

584

585 Table 6 - Comparison of the maximum methane yields of OMW blends reported in the literature for BMP tests.

Substrates	Inoculum adaptation	PP [g l^{-1}]	Maximum methane yield Nml g^{-1}[TVS][7]	[Nml g^{-1} [CODadded]][8]	Notes	Authors
OMW[1]+WW[2]	No	n.a.	n.a.	214	35°C	Fontoulakis et al. (2008)
OMW[1]+WW[2]	No	n.a.	n.a.	301	55°C	
OMW[1] (40%)+PW[3] (60%)	Yes	n.a.	480	297	-	Kougias et al. (2010)
OMW[1] (40%)+PW[3] (60%)	Yes	n.a.	370*	277 Nml g_l^{-1} [$COD_{removed}$]]	-	Kougias et al. (2014)
OMW[1]	Yes	6.0*	n.a.	11.17 [l [biogas] l^{-1} [digester]]	-	Fezzani and Cheikh (2008)
OMW[1] + OMSW[4] (56 g TS/L.OMW)	Yes	3.0*	n.a.	30.5 [l [biogas] l^{-1} [digester]]	-	
OMW[1] (40%) + AW[5] (60%)	Yes	2.04	n.a.	0.189 [l [biogas] l^{-1} [$COD_{removed}$]]	Toxicity test 37°C	Gannoun et al. (2007)
OMW[1] (70%) + LPM[6] (30%)	Yes	6.83*	55.1 [ml g^{-1} [TS]]	n.a.	-	Khoufi et al. (2015)
OMW[1]	No	0.5	419	353	BMP1 tests	This study
OMW[1]	No	1.0	243	205		
OMW[1]	No	2.0	45	37		
OMW[1]	Yes	1.0	419	275	BMP2 tests	
OMW[1]	Yes	2.0	170	111		

586 Notes: [1]OMW = Olive Oil Mill Wastewater; [2]WW = Winery Waste; [3]PW = Pig Waste; [4]OMSW = Olive Mill Solid Waste; [5]AW = Abattoir Wastewater; [6]LPM = Liquid Poultry
587 Manure; [7]TVS = Total Volatile Solids; [8]COD = Chemical Oxygen Demand; *Adapted data.

FIGURE CAPTIONS

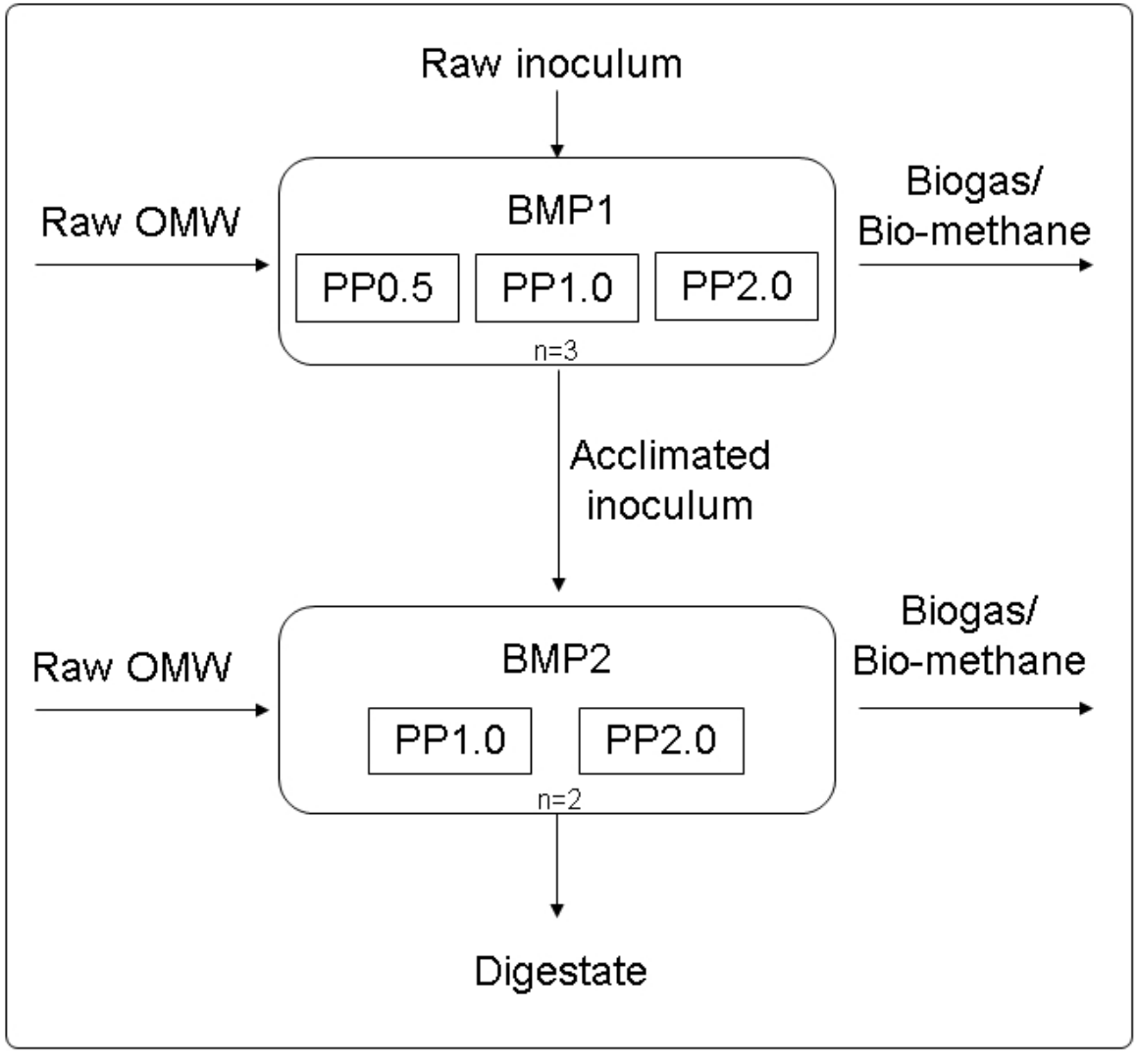
Fig. 1 – Scheme of the BMP experimental design.

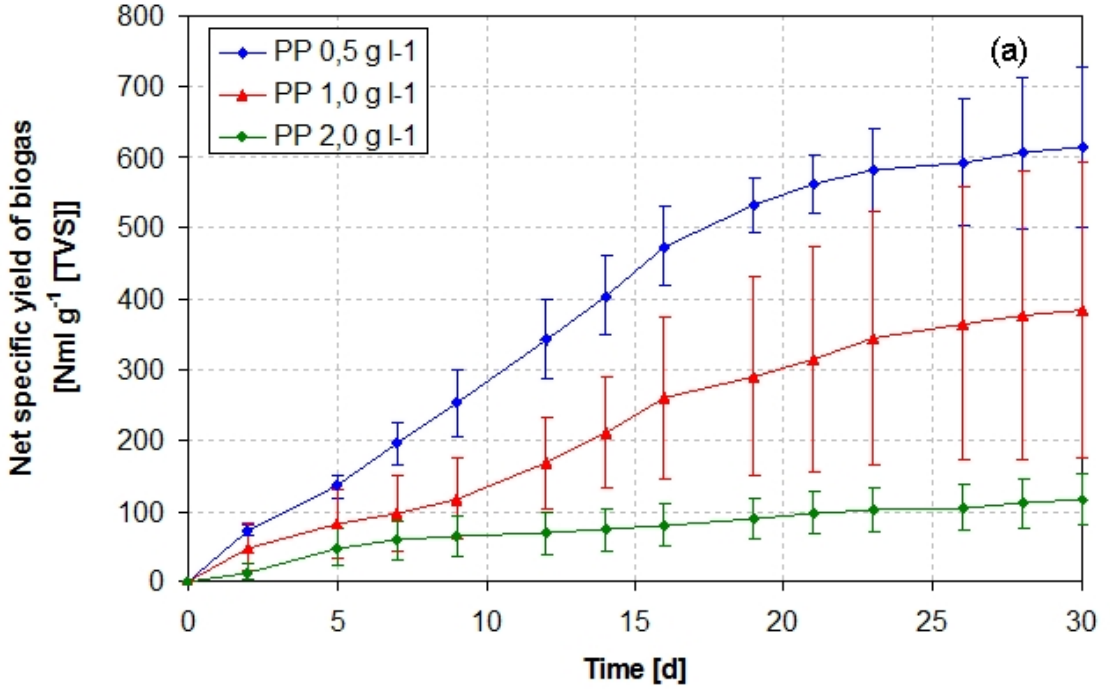
Fig. 2 – Net specific biogas (a) and methane (b) yield in BMP1 tests (mean ± standard deviation).

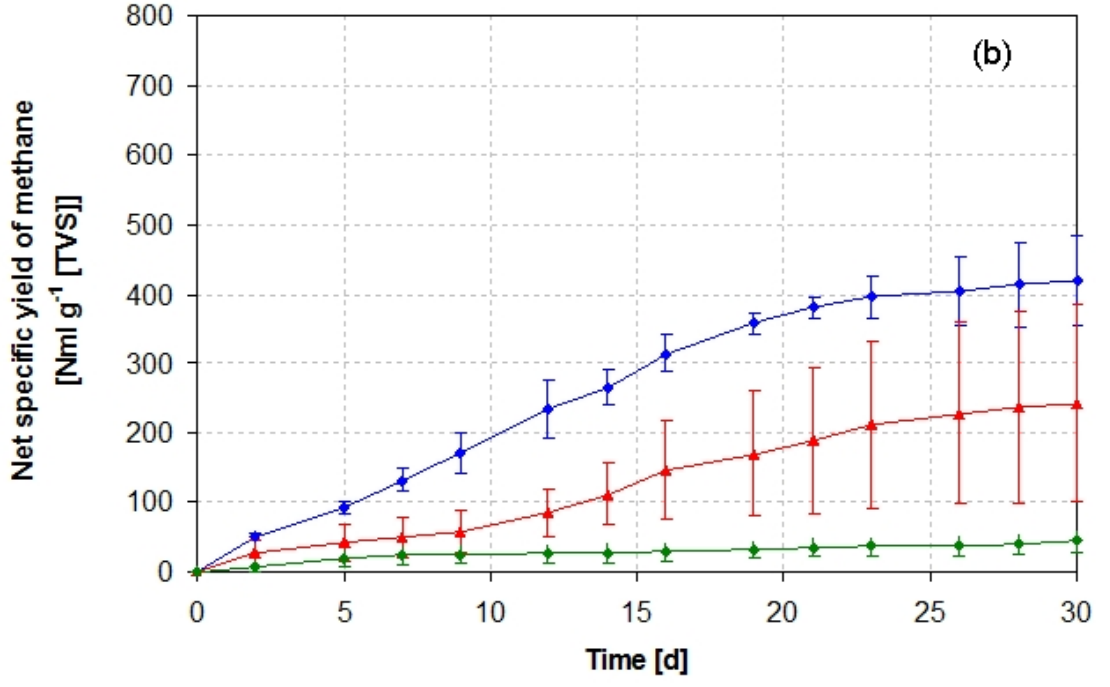
Fig. 3 - Net specific biogas and methane yield in BMP2 tests (mean ± standard deviation).

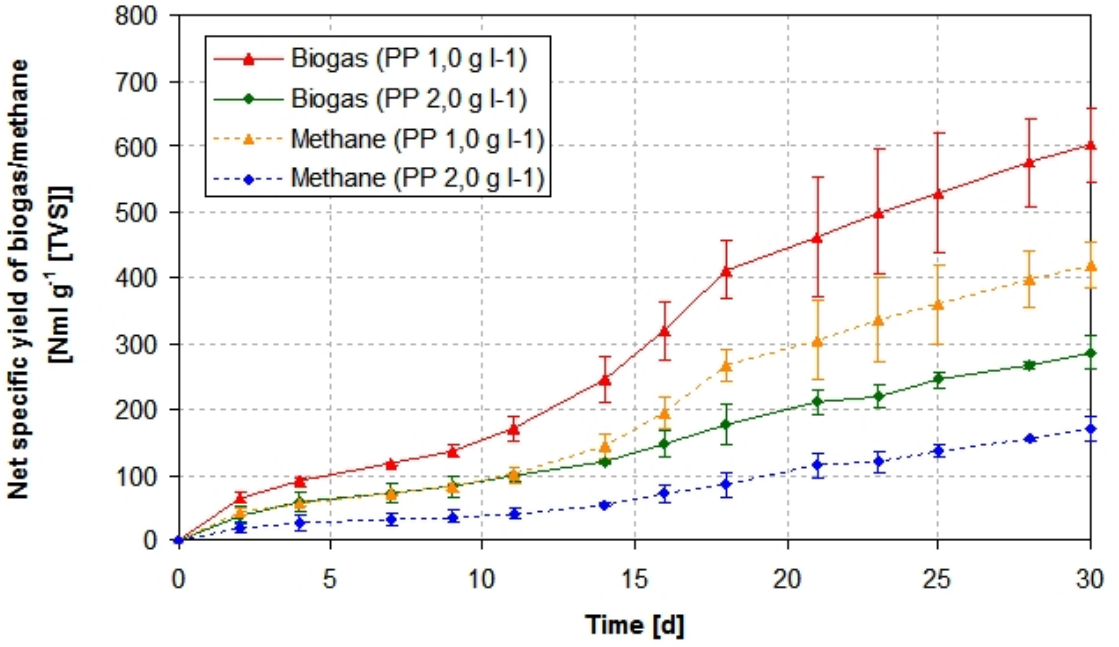
Fig. 4 - Comparison of cumulated specific methane yields measured in BMP1 and BMP2 tests as a function of PP concentrations (mean ± standard deviation).

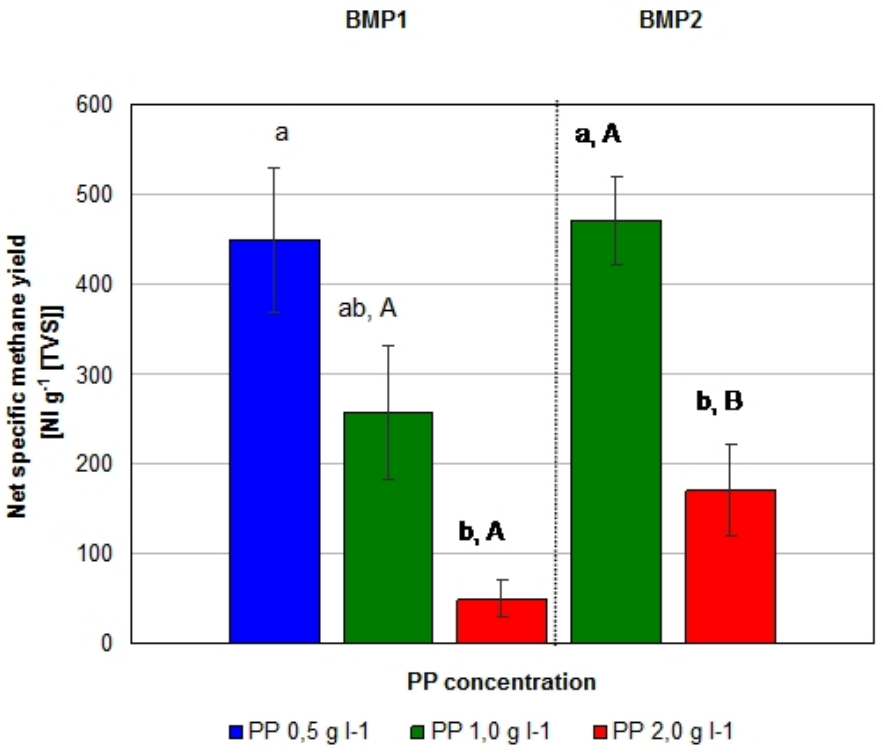
Notes: The different lowercase letters indicate significant differences between PP concentrations within the same BMP series; the different capital letters indicate significant differences between the BMP1 and BMP2 series of blends at the same PP concentration (one-way ANOVA together with Tukey's test for pairwise comparisons at $p < 0.05$).


Raw inoculum
Raw OMW
BMP1
PP0.5
PP1.0
PP2.0
n=3
Biogas/
Bio-methane
Acclimated
inoculum
Raw OMW
BMP2
PP1.0
PP2.0
n=2
Biogas/
Bio-methane
Digestate


PP 0,5 g l-1
PP 1,0 g l-1
PP 2,0 g l-1
(a)
Net specific yield of biogas [Nml g-1 [TVS]]
Time [d]
0
100
200
300
400
500
600
700
800
0
5
10
15
20
25
30


(b)
Net specific yield of methane [Nml g-1 [TVS]]
Time [d]
0
100
200
300
400
500
600
700
800
0
5
10
15
20
25
30


Biogas (PP 1,0 g l-1)
Biogas (PP 2,0 g l-1)
Methane (PP 1,0 g l-1)
Methane (PP 2,0 g l-1)
Net specific yield of biogas/methane [Nml g-1 [TVS]]
Time [d]
0
100
200
300
400
500
600
700
800
0
5
10
15
20
25
30


BMP1
BMP2
600
500
400
300
200
100
0
Net specific methane yield [Nl g-1 [TVS]]
a
ab, A
b, A
a, A
b, B
PP concentration
PP 0,5 g l-1
PP 1,0 g l-1
PP 2,0 g l-1