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# Effect of Heavy Metals in the Performance of Anaerobic Digestion of Olive Mill Waste

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**Abstract:** This study presents an investigation on the effect of heavy metals on the production of biogas during the process of anaerobic digestion (AD) of olive mill waste (OMW). The poisonous effect and the inhibitory influence of Fe, Ni, Pb, Zn, Cu, and Cr on the digestion process are investigated and determined. Biomethanation potential tests are performed for this sake. Adding some of the heavy metals to the AD decreases the efficiency of biogas production and methane concentration and decreases the reduction in the VS, the TCOD, the SCOD, and the organic acid load. A critical increase in the total organic acid and inhibition of methanogenic bacteria was observed due to its toxicity. The toxicity of the heavy metals can be arranged according to increasing order: Cu > Ni > Pb > Cr > Zn > Fe, which leads to rapid poisoning of the active microorganisms. Iron may also exhibit stimulatory effects, but with a low rate and at a certain level. The conclusions of this work are important for the industry and help to understand how to carefully manage the presence of heavy metals in the digestate.

**Keywords:** toxicity; biogas; wastewaters; food industry; biomethanation potential

## 1. Introduction

Waste products, together with biomass, can be an important source of energy. Heavy metals (HM) concentrations can be a problem both in solid and liquid waste treatment. Wastewater treatment processes and poisonous heavy metals (HMs) lead to environmental pollution [1,2]. In particular, referring to the HMs contained in wastewaters, which undergo anaerobic digestion, we have to state that on the one hand, when contained in small concentrations, some heavy metals can also be beneficial and required for the growth of microorganisms; on the other hand, when they are present in

concentrations higher than a certain threshold, they can exert a toxic action. Therefore, HMs toxicity and their accumulation in the industrial processing of wastes represent a serious environmental problem [3].

HMs can be found, for example, in olive mill waste (OMW) effluent in high concentrations [4]. The OMW anaerobic digestion (AD) process generates biogas, which is used as a source of energy [5]. AD has been widely used for OMW with a high content of organic load (OL). In [5], codigestion was performed to enhance substrate biodegradability. Sewage sludge was obtained from the secondary sludge of a wastewater treatment plant situated in the city of Irbid in Jordan. The most effective ratios between olive mill waste and sewage sludge were proved to be about 10% and 90% in volume. In [6], the benefits of codigestion of waste-activated sludge and Organic Fraction of Municipal Solid Wastes (OFMSW) were confirmed at industrial scale. This is a very promising process, given that the disposal of OFMSW in landfills is not recommended in many European MSs, if not forbidden. The industrial tests showed promising results and good performances of the process. Stability was reached after more than 1 hydraulic retention time (HRT). The process proved to be also interesting from the point of view of economics, having a payback period of 3.5 years. In [7], Biomethanation Potential (BMP) tests of source-selected OFMSW were performed in codigestion. Different source-separated organic fractions of municipal solid wastes were tested, as collected from: canteens, supermarkets, restaurants, households, fruit-vegetable markets, and bakery shops. Recently, more efficient AD processes guarantee a high removal efficiency of OL. In [8], glycerol has been used to boost biogas production. Glycerol addition can boost biogas yields, when it is limited to 1% (*v/v*) of the feed volume (the production of methane can be doubled). In [9], thermophilic processes are adopted; tests are performed at both pilot and full scale. It was demonstrated that the thermophilic option can bring an increase of 45–50% in the production of biogas. Metal contents are within the more stringent limit used in Europe for high quality amendment. In [10], modeling is applied to the optimization of anaerobic digestion. Regardless, to have an optimized process, the concentration of HMs in the substrate should be within certain limits. These limits are not clear for all the possible anaerobic digestion substrates and in particular, for olive mill wastes. The aim of this paper is to assess them. These are an important parameters to control the quality of the AD process. In the AD process, metabolism and growth of microorganisms within the substrate play a paramount role in reducing OL and convert it into methane (CH<sub>4</sub>) and carbon dioxide (CO<sub>2</sub>), through sequential processes. In [11], a detailed analysis of bacteria consortia is performed. In [12], the influence of chlorides on the anaerobic digestion process is studied.

Small concentrations of HMs such as Fe, Ni, and Co are needed to improve the efficiency of biogas production in the AD process [13]. Therefore, they can be considered to have a positive action. On the other hand, Pb, Cu, and As affect negatively the efficiency of the AD process; this is due to the toxic effect exerted on AD microorganisms [14]. Some studies reported that certain HMs ions can inhibit enzymes that are produced by microorganisms, thus HMs like Zn, Cu, Cr, and Pb inhibit the AD process [15].

Some studies report the most used methods to reduce HMs levels in raw wastewaters are: the use of cork as a sorbent [16]; microbial [17] or jute fibers [18]. Critical studies reveal that not all metal-polluted OMW can produce biogas through AD; some strategies were performed to remove the HMs from the feedstock material such as the use of biosorbents [19] and dewatering [20,21]. Paganelli et al. (2002) carried out a study on the use of OMW as an HMs sorbent material; they found that Cu was adsorbed in the range of 5–13.5 mg/g under certain operating parameters [22], while Keskinan et al. (2003) reported that the adsorption was about 10.37 mg/g for Cu, 15.59 mg/g for Zn, and 46.60 mg/g for Pb [23].

According to recent studies, the AD process requires a certain concentration of HMs. The AD process requires, in fact, external electron acceptors. If aerobic respiration uses oxygen as an external electrons acceptor, anaerobic digestion needs alternative external electron acceptors (EAs) [24]. Therefore, for the elimination of electrons released during the OL degradation process, Fe, CO<sub>2</sub>, SO<sub>4</sub><sup>-2</sup>, and NO<sub>3</sub><sup>-1</sup> act as external acceptors. When the concentration of heavy metals increases over a certain threshold, there would be inhibition. We have analyzed in this work, which is this threshold,

based on the initial concentrations of heavy metals in the raw material; these have been increased stepwise to find where inhibition begun.

To the best of authors' knowledge, olive pomace anaerobic digestion with changing HMs content has not sufficiently been analyzed, while other substrates, like wastewaters, swine effluents, poultry manure, and swine manure have attracted the bulk of the research efforts [3]. The problem of wastewaters management is particularly important in China, where high concentrations of heavy metals can be measured also in soils and therefore, in agricultural production and agricultural residues. The area of cultivated land polluted by HMs accounts for 20% of the total agricultural land [25].

## 2. Materials and Methods

### 2.1. Feedstock

All the chemicals used were bought from Sigma-Aldrich, St. Louis, Missouri, USA. The OMW substrate was collected from a three-phase oil extraction that belongs to the olive harvest seasons of 2019; it was collected in a 10-L Jerrycan. OMW was stored at 5 °C until it was used. All OMW samples were prepared to be used in biomethanation potential tests (BMP), according to UNI 5667-13/2000. The substrates were analyzed through a thermogravimetric analyzer (TGA 701, LECO, St. Joseph, MI, USA) to perform proximate analysis; methods are described in Alrawashdeh et al. (2017) [26] and Alrawashdeh et al. (2017) [27]. Proximate analysis is useful to measure ash content, total solids, volatile solids, moisture, and fixed carbon (F.C.). The OMW substrate characteristics are summarized in Table 1.

**Table 1.** Characterization of olive mill wastewater (OMW).

Substrate	Moisture (%)	Total Solids (%)	Volatile Solids (%)	Ash (%)	Fixed Carbon (%)	pH
OMW	86.57 ± 0.8	13.43 ± 1.3	6.5 ± 0.9	4.42 ± 2.5	2.51 ± 1.2	4.9 ± 0.24

The HMs were detected by an atomic absorption spectrophotometer (Optima8000, Perkin Elmer, Waltham, MA, USA) [28]. The OMW samples were oven-dried at 100 ± 1 °C by TGA, then acid-digested according to Liu et al. (2001) [28]. The analysis was repeated 3 times. The total chemical oxygen demand (TCOD) and the soluble chemical oxygen demand (SCOD) were obtained according to Apha (1998) [29]. The polyphenols concentration was detected by a spectrophotometric Folin–Ciocalteu method, according to Alrawashdeh et al. (2019) [26,30]. The heavy metals concentrations of OMW are reported in Table 2.

**Table 2.** Heavy metals concentration in the olive mill waste and in the inoculum, plus other parameters.

Parameters (mg/L)	OMW	Inoculum
Fe	1.45	4504
Ni	0.041	<0.001
Pb	0.17	332.9
Mn	<0.001	960.6
Zn	0.29	28.77
Cu	1125	0.28
Cd	<0.001	197
Total COD	116.62 ± 0.61	88.9 ± 1.08
Soluble COD	61.53 ± 2.16	37.01 ± 0.04
Polyphenols	4.51 ± 1.13	-
Organic acids load	190	-

The organic acids concentration was detected with PerkinElmer Altus™ HPLC, Waltham, MA, USA. Table 2 shows the average composition of olive mill waste, as produced in the Jordan industry.

## 2.2. Experimental Setup

Fourteen anaerobic reactors (ARs) of 2l volume were prepared. ARs were realized in transparent PVC, which were provided with two sealed valves. One of the valves was used to collect the biogas, while the second was used to introduce the OMW substrate. Six of the ARs were used to investigate the effect of HMs on the biogas production, and one AR was used for OMW, without any addition of HMs as a control; each test was repeated 2 times [30].

The substrate was inserted in the reactor with an inoculum to substrate ratio of about 20% in volume [30]. Activated sludge (AS) collected by a local wastewater treatment plant was used as the inoculum; it was kept for two months in incubation to obtain active bacteria. The inoculum's main characteristics are:  $80 \pm 1.1$  g/L of TS,  $62 \pm 2.7$  g/L of VS, and pH about  $7.2 \pm 0.92$ . As it is shown in Figure 1, the AD system consisted of the APP reactor placed in a water bath, instrumented with two outlets—one slot to collect biogas through a silicone tube connected to another vessel which contained a NaOH 2% solution, while another slot was occupied with a silicon tube to introduce the OMWC substrate. There was also a tube from the bottom of the second vessel which contained NaOH, through which the biogas pushed the NaOH solution into another graduated vessel, which was used to measure the volume of the daily produced biogas (see [26]).

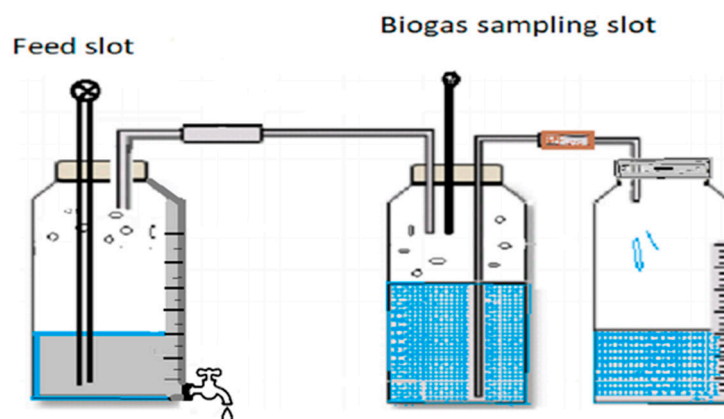


Figure 1. Anaerobic digestion reactor configuration.

ARs were incubated for a 20-day period to achieve steady state operation and they were magnetically mixed; the inoculum (AS) to substrate ratio was about 20% and the vessels were sealed with silicon. Each AR was filled with 325 g of OMW substrate, 65 g of inoculum, and 10 mL of water; these quantities were chosen to achieve a substrate in the range of 90% of humidity. ARs were purged by nitrogen to obtain the necessary anaerobic atmosphere. All the ARs were operated at  $30 \pm 2$  °C

The ARs were thereafter fed continuously with the OMW substrate. The feeding procedure involved withdrawing 50 mL effluent of OMW and introducing the same quantity into the reactor. The experiment continued for two months. At the beginning, the feeding process was implemented without any addition of HMs for 20 days. After 20 days, each AR was fed with OMW with a specific concentration of HMs. The volume of generated biogas was obtained by measuring the volume of the liquid NaOH solution displaced by the biogas, as previously described [31]. The methane percentage was analyzed using a gas chromatograph (GC), according to Alrawashdeh et al. (2017) [26].

After the steady state was reached, the pH for each reactor was carefully monitored. After 20 days, a particular HM was fed to each AR. During the digestion period, the HMs were added with two techniques:

1. For Fe, Ni, Pb, Zn, Cu, and Cr, a stepwise technique was used.
2. For Mn and Cd, the pulse feeding technique was used, because the concentration level of Mn and Cd was very small  $<0.001$ .

Mn and Cd are not discussed in the results section because their effect on anaerobic digestion was reduced.

A certain quantity of HM was added to each feed batch of OMW; it was mixed and homogenized with the feed and the mixture was inserted once every 3 days. The dosages of HM used in this study are presented in Table 3. The whole test lasted for 80 days and every 3 days after the feeding, the influence of the dose on the AD process was investigated. The system was monitored during the tests, and the volume of generated biogas and the methane concentration indicated the system's stability. The pH values were controlled by adding KOH to each AR to increase the pH value to grant optimal conditions to the bacteria (subsequently, in the range of 7–7.5). BF \* means the ratio between the quantity of HM in the dose added with the new feed into the reactor and the quantity of HM already present inside the material digested in the reactor. The quantities of heavy metals are increased with respect to the initial concentration of HMs in the olive mill waste.

**Table 3.** Quantity of heavy metals in the feed olive mill waste substrate added to the reactor.

HM	HM Concentration in Feed Substrate (mg/L)	BF *	HM	HM Concentration in Feed Substrate (mg/L)	BF *
Fe	0.362	0.25	Zn	0.072	0.25
	0.725	0.5		0.145	0.5
	1.087	0.75		0.217	0.75
	1.45	1		0.29	1
Ni	0.010	0.25	Cu	281.25	0.25
	0.020	0.5		562.5	0.5
	0.031	0.75		843.75	0.75
	0.041	1		1125	1
Pb	0.042	0.25	Cr	0.173	0.25
	0.085	0.5		0.346	0.5
	0.127	0.75		0.519	0.75
	0.17	1		0.692	1

$$\text{BF}^* (\text{Background factor}) = \text{HM}_{\text{feed}} / \text{HM}_{\text{digester}}$$

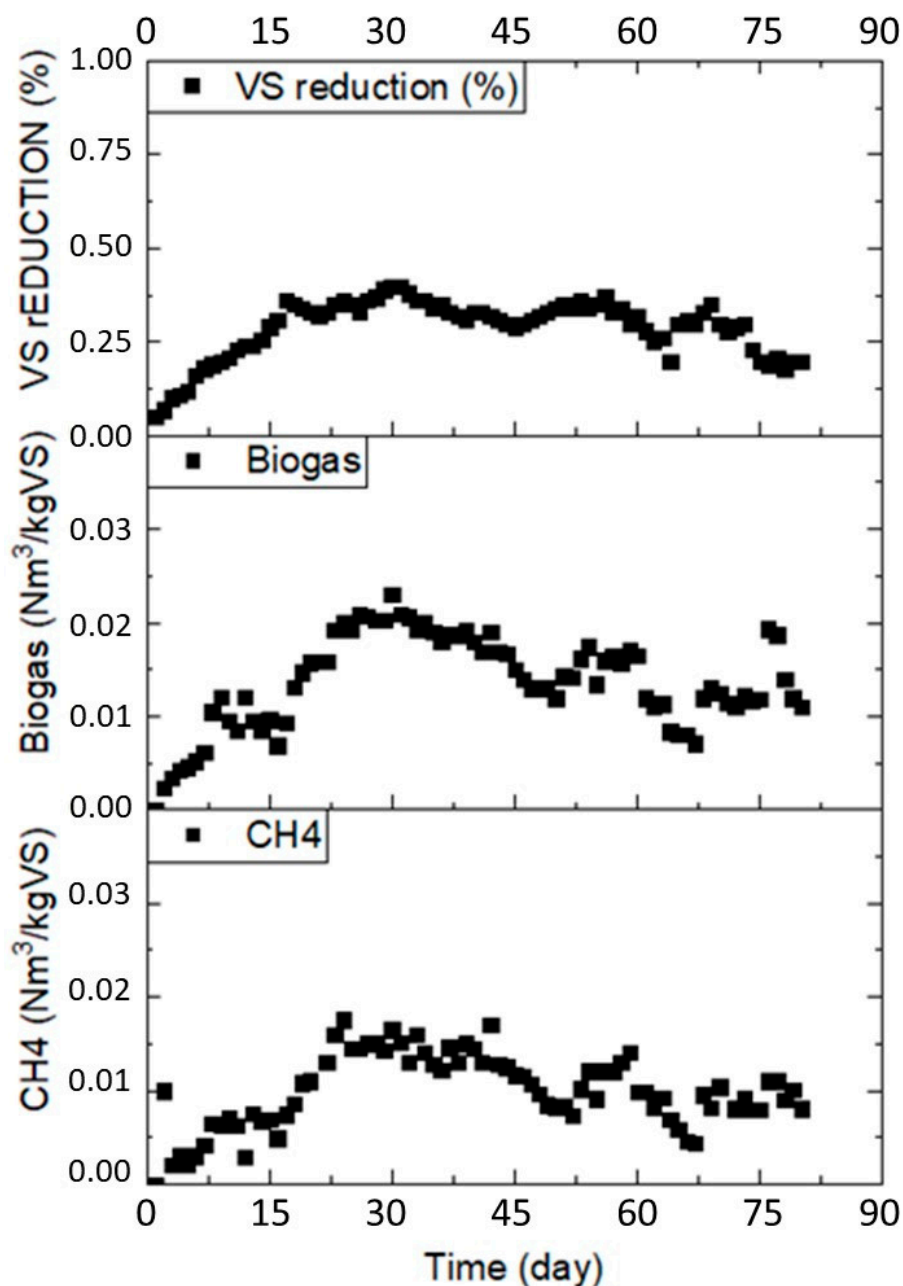
### 3. Results and Discussion

#### 3.1. Anaerobic Digestion Tests without Any Heavy Metal

The results showed that the production of biogas from the substrate containing OMW, without any addition of HMs, started after 2 days and it increased gradually until the 22nd day. Then, it became steady until the 50th day; after that, it oscillated until the end of the test. The cumulative production of biogas and methane volumetric concentration were  $0.426 \text{ Nm}^3/\text{kg VS}$  and 64.6%, respectively, while the TCOD reduction and the SCOD reduction were in the range of  $30 \pm 0.30$  to  $36 \pm 1.40$  and  $32 \pm 0.91$  to  $28 \pm 2.30 \text{ g/L}$ , respectively. The daily production of biogas and methane and the VS reduction for the AR, without any addition of HMs, are illustrated in Figure 2.

#### 3.2. Anaerobic Digestion Tests with the Addition of Heavy Metals

The effect of increasing doses of HMs on the behavior of the AD process was also monitored. At the start of each test, a drop in system performance was noted. In general, performance increased when adding Fe. After the acclimation period, the ARs were fed with 50 mL of OMW substrate containing the specific quantity of HMs indicated in Table 2. The performance of each AR was measured monitoring biogas production, methane concentration, VS reduction, SCOD reduction, and TCOD reduction.



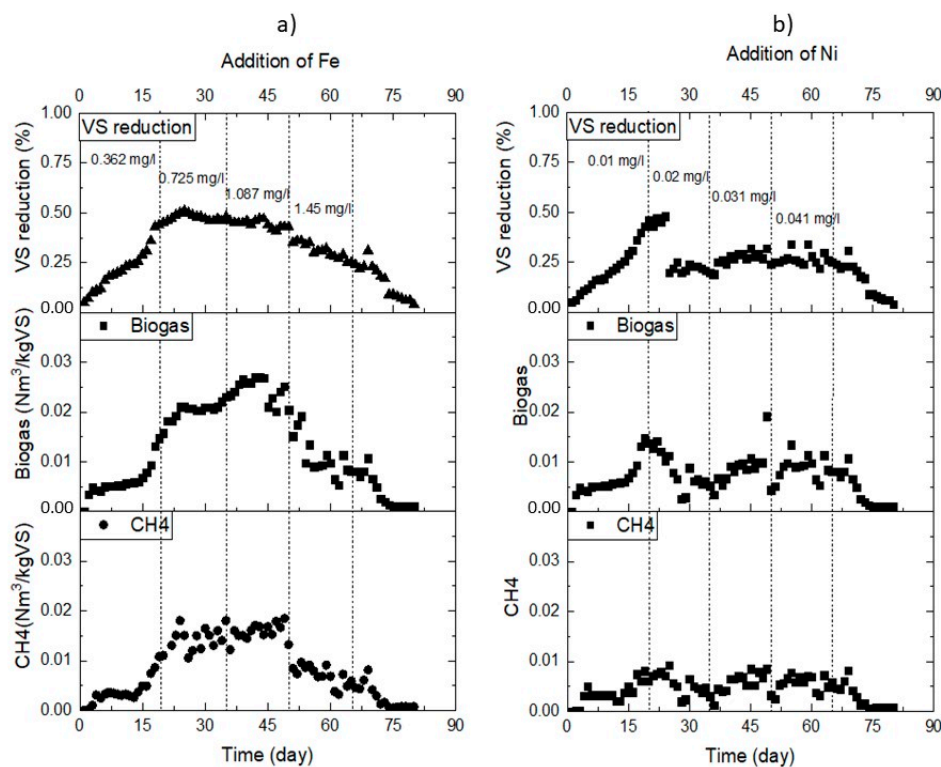
**Figure 2.** Performance of olive mill waste without any addition of heavy metals.

### 3.2.1. Addition of Iron (Fe) and Nickel (Ni)

The impact of an incremental dose of Fe on the anaerobic digestion process was analyzed by increasing the dose of Fe from 0.362 to 1.45 mg/L, in a stepwise fashion. The effect of each dose of Fe was monitored for 15 days. We noted that Fe addition increased biogas production and CH<sub>4</sub> production in the range of 0.013 and 0.021 Nm<sup>3</sup>/kg VS, respectively; the corresponding VS reduction was increased by 10%. The impact of Fe addition is shown in Figure 3. The addition of Fe improved AD performance until the 70th day. Then, the process of digestion began to deteriorate and this can be seen clearly through Figure 3a. The addition of Fe increased and accelerated the initial exponential biogas yield and methane production rate. The same results for Fe addition were noted by Kim et al. (2002); they reported that the addition of certain HMs to the feed materials in AD has been found to increase biogas and methane production and the removal efficiency of propionate when high levels of volatile fatty acid were experienced [32]. Gonzalez-Gil et al. (1999) also concluded that the addition of FeCl<sub>3</sub> caused an increase



in the concentration of methane in the biogas to values which were higher than 60 v% [33]. Dealing with the influence of increasing doses of Ni, from 0.01 to 0.041 mg/L, on the anaerobic digestion process, this resulted in a performance decrease, as shown in Figure 3b. The digestion process continued, but at a low rate until the 70th day, and afterward, it stopped. The biogas production of the AR with Ni addition, compared to the AR without any addition, decreased to a value of 0.083 Nm<sup>3</sup>/kg VS, but the concentration of CH<sub>4</sub> was higher. The VS reduction was in the range of 19w%. This result is in agreement with what was reported in Kumar et al. (2006) [34], who analyzed the effect of HMs on potato waste anaerobic digestion. They reported that the AD process improved in terms of biogas production through the addition of HMs at concentrations of 2.5 mg/L (wet basis). The biggest increase was obtained adding Cd, followed by Ni, and then, by Zn.

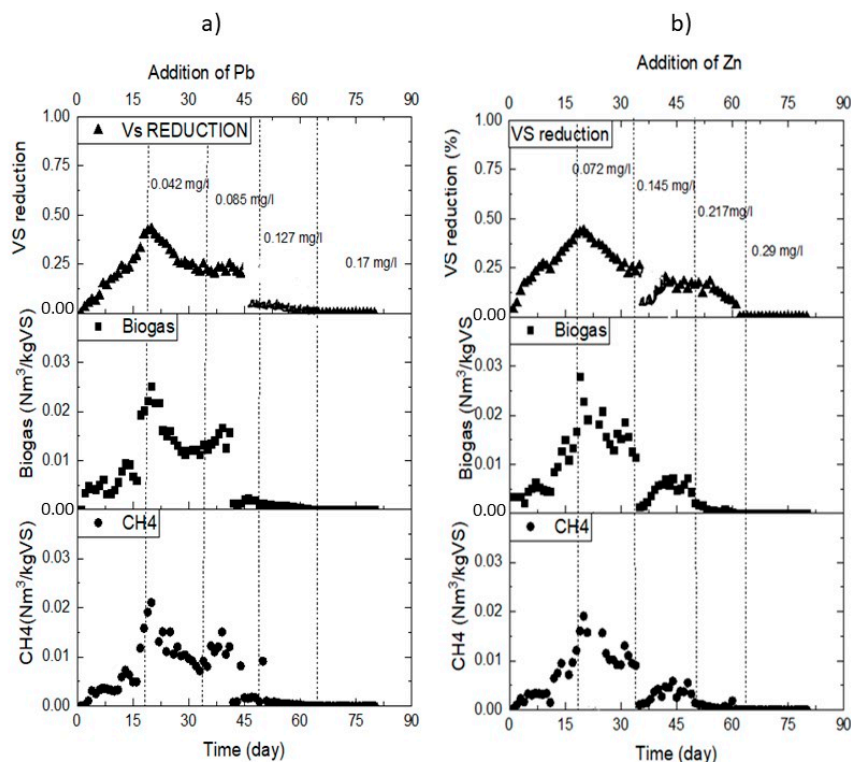


**Figure 3.** (a)—Impact of the addition of Fe on anaerobic digestion performance; (b)—Impact of the addition of Ni on anaerobic digestion performance.

### 3.2.2. Addition of Lead (Pb) and Zinc (Zn)

During the addition of Pb, the impact of the increase in lead doses from 0.042 to 0.17 mg/L was tracked continuously (Table 2). The impact of each dose was monitored for 15 days. In comparison with the anaerobic digestion without any addition of HM, Pb feeding up to 0.042 mg/L reduced biogas and CH<sub>4</sub> production by 0.234 and 0.164 Nm<sup>3</sup>/kg VS, respectively (approximately half of the production of the AR without the addition of HMs). The corresponding VS reduction was 22%, the quantity of TCOD and SCOD increased to 17 ± 1.60 in the first test and to 14.5 ± 1.10 g/L in the second test and to 20 ± 0.21 in the first test and to 13 ± 0.95 g/L in the second test, respectively. With the dose of 0.127, the anaerobic digestion performance decreased sharply and then, stopped after the dose of 0.17, as shown in Figure 4a. The continuous feeding of the system with Zn, from a dose of 0.072 up to a dose of 0.29 mg/L, reduced the VS reduction by 30w%, and increased the TCOD and the SCOD to 21 ± 1.20 in the first test and 17.25 ± 2.10 g/L in the second test and to 23 ± 0.73 in the first test to 15 ± 1.05 g/L in the second test, respectively, compared to anaerobic digestion test without any addition of HMs. In addition, as a result of Zn addition, the reduction in biogas production resulted to be of 40% with respect to the base case, as shown in Figure 4b. Biogas production stopped after the dose of 0.29 mg/L.

The addition of Zn delayed degradation during the acidogenic–anaerobic treatment; this matches with what was reported by Lang et al. (2007) [35] and Aziz et al. (2004) [36].



**Figure 4.** (a)—Impact of Pb on the anaerobic digestion performance; (b)—Impact of Zn on the anaerobic digestion performance

### 3.2.3. Addition of Copper (Cu) and Chromium (Cr)

The Cu dose in the feed was increased in the range of 281.25 to 1125 mg/L. After adding 281.25 mg/L of Cu, a reduction in the removal of the VS was noted. The anaerobic digestion process reached its maximum drop after 18 days and the system showed further inhibition by adding 562.5 mg/L of Cu. The reduction in VS degradation and biogas production reached 70% and 77%, respectively, during the addition of the dose of 281.25 mg/L. Biogas production decreased in total by 0.233 Nm<sup>3</sup>/kgVS, while the TCOD and SCOD increased by 55% and 60%, respectively. The performance of Cu addition is illustrated in Figure 5a. The results indicated that the increasing concentration of Cu exerted an inhibition of methanogenic activity, which matches with Bartacek et al. (2008) [37].

By adding increasing doses of Cr, ranging from 0.173 to 0.692 mg/L, a remarkable reduction in the performance of AD was exhibited. When the concentration of Cr in the feed was up to 0.519 mg/L, as compared to the AR without any addition of HMs, the volume of the daily generated biogas, CH<sub>4</sub> production, and VS reduction were reduced by 59%, 56%, and 55%, respectively. The Cr performance is shown in Figure 5b.

However, we noted that Cu and Cr at a certain concentration effectively inhibited biomethane production in the OMW, but stimulated it at the lower concentration (lower than BF = 0.25). This result corresponds with Mishra et al. (1999) [38].

Figure 6 shows the comparison between the performance of the digestion of the OMW substrate without HMs and the digestion of the OMW with the addition of HMs in terms of the cumulative biogas and the CH<sub>4</sub> percentage. As illustrated, the addition of Fe improved the efficiency of the AD process, while the other HMs' addition led to a decrease in the efficiency of the AD process. The figure below shows biogas production according to HMs addition. The inhibiting action exerted from the addition of the elements is classified according to the following order: Fe > Zn > Cr > Pb > Cu > Ni.



The methane contained in the biogas, according to the HMs addition, was influenced in this order Fe > Zn > Cr > Pb > Ni > Cu. The percentage of CH<sub>4</sub> contained in the biogas was 0.646%, 0.675%, 0.615%, 0.607%, 0.591%, 0.568%, and 0.528% for the OMW without any addition of HMs and with the addition of Fe, Cr, Pb, Zn, Ni, and Cu, respectively.

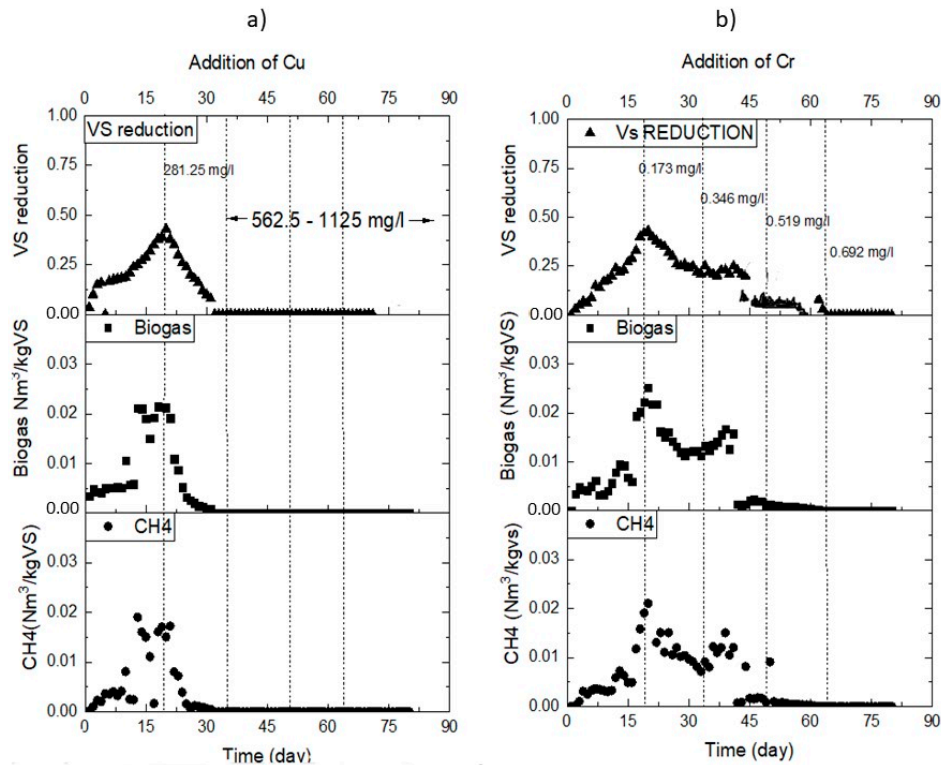


Figure 5. (a)—Impact of Cu on the performance of AD; (b)—Impact of Cr on the performance of AD.

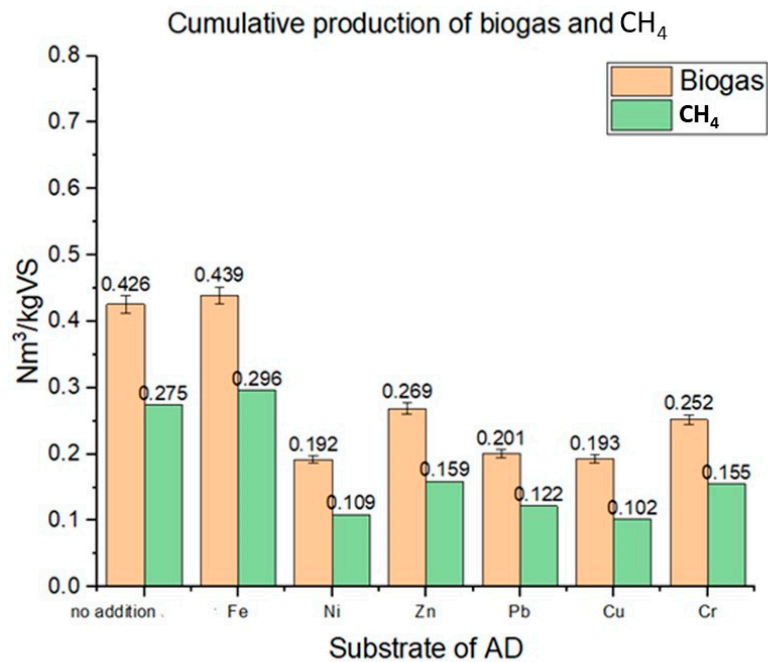


Figure 6. Cumulative production of biogas and methane for raw material without any addition of HMs and with the addition of a certain HM.

Once the HMs concentration increased more than a certain level, this led to inhibition of the AD process, in agreement with what was reported in the anaerobic digestion tests documented in [39] and in the biosorption and bioaccumulation tests reported in [40].

The level of inhibition of a HM can be determined as the one which leads to a radical decrease in the generated biogas and increases the toxicity for the AD process. A toxic effect was identified as that at which the yield of biogas was decreased by 50% with respect to the reference value. The inhibitory level and toxicity level of HMs are reported in Table 4. Those agree with the levels specified in the study of Zheng Bo, Y. et al. 2014 [13]. In addition, the authors in [17], which studied the biosorption process, report similar values. On the effect of HM on the anaerobic digestion process, the authors in [41,42] also report significative data. Mudhoo and Kumar (2013) reported that the HMs like Cu, Ni, Zn, Ca, Cr, and Pb are inhibitory and under certain conditions toxic in biochemical reactions, depending on their concentrations. Most studies have demonstrated that the toxic level of HM is attributable to the disruption of the function of enzymes [43].

**Table 4.** Inhibiting level and toxic threshold of olive mill waste during the anaerobic digestion process.

HM	Inhibiting Level (mg/L)	Toxic Limit (mg/L)
Fe	>0.87	>1.45
Ni	>0.02	>0.041
Pb	>0.85	>0.127
Zn	≥0.145	≥0.29
Cu	≥281.25	≥562.5
Cr	≥0.173	≥0.692

The total reduction in the organic acids load, in the TCOD, in the SCOD, and in polyphenols during the AD tests, with the addition of HMs, are reported in Table 5. This shows how the value of TCOD, which is removed from the substrate with the addition of Fe, can induce a stable TCOD conversion rate, while Cu addition achieves lower stability and reduces the TCOD reduction. We observed the same trends for SCOD, VS, polyphenols, and total organic acids.

**Table 5.** Chemical characteristics of the effluent of the anaerobic digestion process for all tests.

Fed Substrate	Organic Acids Load <sub>effluent</sub> (mg/L)	TCOD <sub>effluent</sub> (mg/L)	SCOD <sub>effluent</sub> (mg/L)	Polyphenols <sub>effluent</sub> (mg/L)
OMW *	208 ± 2.5	138.07 ± 1.53	72.61 ± 0.19	0.510 ± 0.04
Fe	204.3 ± 1.17	140.1 ± 0.96	71.3 ± 0.35	0.507 ± 0.11
Ni	187.6 ± 2.82	129.5 ± 1.43	67.9 ± 1.28	0.476 ± 0.14
Zn	200.1 ± 0.57	135.2 ± 3.11	69.4 ± 1.02	0.480 ± 0.10
Pb	197.8 ± 3.04	131.4 ± 1.66	68.3 ± 1.7	0.463 ± 0.03
Cu	195.5 ± 1.45	128.1 ± 2.05	66.53 ± 0.89	0.458 ± 0

\* Without any addition of HM.

Overall, by tracking the performance of all HMs, it is clear that the activity of methanogenic bacteria decreased with the increase in the concentration of HMs. The toxicity of each HM was evaluated by the reduction in CH<sub>4</sub>, as compared to the controls. The result shows that the toxicity of HMs had the following descending order: Cu > Ni > Pb > Cr > Zn > Fe. This matches with what is reported by Fang (1997) [44].

This result disagrees with Baath (1989); he reported that the toxicity increases as follows: Pb > Zn > Cu > Cd, depending on numerous abiotic and biotic factors [45]. These results are mostly related to the chemical binding of HMs to the enzymes and their capacity to influence microorganisms metabolism. This leads to delay in the activity of the enzymes, also according to Mata-Alvarez et al. (2000) [46], who performed an overview of the perspective and achievements on organic solid waste anaerobic digestion. Special attention was focused on optimal conditions and inhibition. Bayer et al. (2007) [47]

also focused analysis on the hydrolysis phase as a key phase in anaerobic digestion and as the part where more likely, inhibition can be exerted. Cirne et al. (2007) [48] underlined once again the effect of the hydrolysis process on lipids anaerobic digestion, focusing in particular on volatile fatty acids production and on the inhibiting effect of long chain fatty acids production. Li and Fang (2007) [49] analyzed the toxic effect of heavy metals on H<sub>2</sub>-producing bacteria, through dark fermentation tests. They demonstrated that H<sub>2</sub>-producing sludge exhibited, in general, higher resistance to metal toxicity than methanogenic granular sludge. Besides this, they confirmed the order of magnitude of the toxicity of different metals presented also in this work. Wani et al. (2012) examined instead the toxic effects of heavy metals in plant organisms [50].

#### 4. Discussion

In this paper, different concentrations of heavy metals were used to simulate different compositions of olive mill waste, which already contain them. Therefore, the aim was to assess how the variability on heavy metals content can affect the final results of the anaerobic digestion. This was assessed based on the variations of the produced biogas, its composition, and the destruction of volatiles, organic acids load, TCOD, SCOD, and polyphenols.

The limitations of the study are that a complete analysis of the microbial community is missing, so we actually do not know the effect of the heavy metals on the single microorganism, but to perform this kind of analysis, the methods should be also changed significantly.

The results of this analysis are useful to optimize the anaerobic digestion process of olive mill waste effluents and it is one of the first contributions available in the literature in this sense. The fact that low concentrations of HMs promote the anaerobic digestion process is recognized also in a recent review [3], in which it is stated that low doses of Cu<sup>2+</sup> and Cd<sup>2+</sup> serve as cofactors in the catalytic center of cellulase and stimulate enzyme activity. On the other hand, high contents of Cd<sup>2+</sup> and Cu<sup>2+</sup> inhibit enzyme activity by disrupting protein structures.

The effect of heavy metals on AD process are proposed in Table 6.

**Table 6.** Effects of heavy metals on the anaerobic digestion process [3].

Heavy Metal Type	Effect on Anaerobic Digestion
Cu [3]	Has a negative effect on hydrolysis which, in the case of cellulose, is catalyzed by cellulases. The impact is concentrated on the spatial structure of the enzyme. High concentration of Cu can inhibit also methanogenic bacteria.
Ni [3]	High concentrations of nickel also have negative effects on cellulases and methanogenic bacteria.
Zn [3]	The influence of zinc on bacteria is not clear still and its inhibiting effect seems to be quite reduced. A slight negative effect can be exerted on methanogenic bacteria growth.
Cd [3]	Cadmium has high toxicity for methanogenic bacteria.
Fe [3]	Iron can have a positive effect on anaerobic digestion acting on sulfide and reducing its negative effect. Fe generally increases methane production acting on proteolytic enzymes, sucrases, and cellulases. Fe is also important in stimulating the formation of cytochromes and ferredoxin (Fd), which are vital for electron transportation.
Pb [51,52]	Pb was proved to negatively affect bacteria activity and also pH. Pb can damage microbial cell membrane and also take part in the microbial metabolism, influencing it in a negative way.
Cr [53]	The effect of chromium depends on its form (either VI or IV) and on the stability of the waste. The toxicity of chromium for bacteria still needs to be assessed further.

#### 5. Conclusions

Among the stress factors which may inhibit a proper anaerobic digestion process and limit biochemical reactions, heavy metals effects were discussed in this study. A significant decrease in

the performance of the anaerobic digestion process, biogas yield, CH<sub>4</sub> concentration, VS, TCOD, and SCOD was detected. The main quantitative results of this study show that HMs may be inhibitory, toxic, or even stimulatory to the anaerobic digestion process. These impacts depend on the HMs concentration. The effects of HM on the acetogenic and methanogenic stages were examined through CH<sub>4</sub> concentration, VS reduction, and the organic acids load reduction. It was concluded that the toxicity of the HMs can be arranged according to the increasing order Cu > Ni > Pb > Cr > Zn > Fe, which lead to rapid poisoning of the activity of microorganisms. This study shows that the concentration of Fe, Zn, Cr, Pb, Ni, and Cu can safely improve the AD process (in terms of increasing biogas and methane production and increasing TCOD, SCOD, VS, and polyphenols removal) if they are lower than 2.9, 0.335, 1.211, 0.297, 0.082, and 1406.25 mg/L, respectively. Therefore, it is recommended to control the level of HMs in the digestion process for biogas production. Biogas production and methane content according to HMs addition were arranged ascendingly: Fe > Zn > Cr > Pb > Cu > Ni and Fe > Zn > Cr > Pb > Ni > Cu, respectively. Recommendations are focused on a more careful analysis of the substrate and the use of adsorption and retention systems to reduce the concentration of HMs in the substrate below the above reported toxic levels.

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