Bull Environ Contam Toxicol (2010) 85:432–436
DOI 10.1007/s00128-010-0106-x

Comparative Assessment of Olive Oil Mill Effluents from Three-phase and Two-phase Systems, Treated for Hydrogen Production

A. Rouvalis · J. Iliopoulou–Georgudaki

Received: 9 December 2009 / Accepted: 13 September 2010 / Published online: 24 September 2010

Abstract By-products of a two-phase and a three-phase olive oil mill process treated in an anaerobic fermentation system for hydrogen production, were evaluated by three bioassays: the zebrafish *Danio rerio* embryo test and two microbiotests, Thamnotoxkit F and Daphtoxkit F^{TM} pulex. Samples from both processes were classified as "very toxic" with LC_{50} values ranging from 1.52% (*T. platyurus* 24 h test) to 4.48% (*D. pulex* 48 h-LC_{50}). Toxicity values were differently correlated to physicochemical parameters showing different degree of influence. The treated effluents of both process systems remained very toxic showing the necessity for further treatment, aiming to environmentally safe discharges.

Keywords Toxicity · Olive oil mill effluent · Thamnocephalus platyurus · Daphnia pulex · Zebrafish · Anaerobic digestion

Over the past years anaerobic process has been widely used for the treatment of industrial and agricultural wastes. One of the most complex agricultural wastes are those generated from olive oil milling, which are usually stored in disposal ponds or rejected to aquatic (rivers, sea) and/or terrestrial environments with no treatment, resulting to a common problem in the olive oil producing countries. The most widespread technology for olive oil extraction consists of the continuous centrifugation system, which is either a three-phase process or a two-phase. In the former sytem three phases are produced: olive oil, a solid residue and an aqueous liquor (Sayadi et al. 2000). The two-phase process that is an emerging technology, but not common in Greece, generates olive oil as the liquid phase and a semi-solid organic waste (Koutrouli et al. 2006). It is considered environmentally friendly in comparison to the three-phase system as it requires a lower water volume during the process, but the effluent is a slurry olive mill solid waste that is not easily managed as the polluting load is more concentrated (Kapellakis et al. 2008). The by-products from both systems consit a major environmental problem that has been studied by many researchers. The olive oil mill wastewaters (OMW) are the aqueous effluent obtained from the three-phase olive oil extraction process and are characterized by a high organic load, suspended solids and a number of organic compounds with monoaromatic and polyaromatic molecules such as phenols, responsible for the toxicity that has been ascribed to this effluent (Rouvalis et al. 2004; Sayadi et al. 2000). The olive mill semi-solid organic waste (OMSW), which is generated in large amounts from the two-phase processes, has a high content in organic matter, nutrients and phenolics that easily range from 12 to 26 g/kg (Aranda et al. 2007).

One of the most promising treatment technologies regards the biological anaerobic digestion (Koutrouli et al. 2006). It has been reported that OMW and OMSW can be used as an alternative substrate for biological hydrogen production due to the high composition in hydrocarbons (Koutrouli et al. 2006; Eroğlu et al. 2004), resulting to an effluent with reduced BOD and COD concentrations. The objective of the current study was to evaluate the treated effluents generated from an anaerobic fermentation system in terms of environmental acceptability and bioremediation and to determine the potential differences between the two

A. Rouvalis · J. Iliopoulou–Georgudaki (✉)
Department of Biology, Section of Animal Biology,
Unit of Environmental Management, Pollution
and Ecotoxicology, University of Patras, 26500 Patras, Greece
e-mail: j.iliopoulou@upatras.gr

processing systems. For this purpose the toxicity evaluation was applied as it is an important index in waste characterization (Mekki et al. 2008). The toxicity of the treated effluents was estimated by two freshwater invertebrates (*Daphnia pulex* and *Thamnocephalus platyurus*) as well as by zebrafish (*Danio rerio*) embryos. Based on the results of the toxicity tests and the probable relations to several physical and chemical properties of the final effluent, conclusions could be derived about the efficiency of the specific treatment technology in the remediation of olive oil mill wastewaters.

Materials and Methods

The OMW were collected from the separators of a three-phase centrifuge system unit located in the Achaia Prefecture, Greece and stored at a temperature below 6°C in the Laboratory of Biochemical Engineering and Environmental Technology (Department of Chemical Engineering, University of Patras) for treatment. Additionally, the OMSW was collected from a two-phase unit located in Kalamata (Messinia Prefecture), Greece, and stored accordingly.

Both effluents were treated in a laboratory scale anaerobic continuous stirred-tank reactor (CSTR) for hydrogen production in the cooperative laboratory of Biochemical Engineering and Environmental Technology (Department of Chemical Engineering, University of Patras, Greece). The reactor operated at a mesophilic temperature with a thermostatic jacket set at 35°C. The reactor was fed separately either with OMW or with OMSW, both diluted with tap water (1:4). The experiments took place as described by Koutrouli et al. (2006). During the operation period, 23 samples from treated OMW and 16 samples from treated OMSW were collected, once steady state conditions were achieved. Each sample was divided and stored at −20°C to be used for the bioassays, and at 4°C to be used for the physicochemical analyses.

All samples were analyzed for specific physichochemical parameters, namely for the ions nitrate, nitrite, ammonium, phosphate, sulphate, and total chloride, using a Hach spectophotemeter DR2800 which is based on Standard Methods for the Examination of Water and Wastewater (APHA 1989). Total suspended solids (TSS) and pH were measured with a portable Multiparameter Hach device (Series Ion 156). Tannin and lignin were determined according to the "Standard Methods for the Examination of Water and Wastewater" (1989). The phenolic compounds were determined spectrophotometrically using the Folin – Ciocalteu method (Box 1983). Calibration was carried out using syringic acid as a standard and values are reported by reference to a standard curve ($y = 10.473x$, $R^2 = 0.993$). Absorbance was measured at 760 nm.

For the toxicity evaluation of the treated effluent samples three toxicity tests were used; two microbiotest kits Daphtoxkit F^{TM} pulex (2006) and Thamnotoxkit F (2006), utilizing the cladoceran *D. pulex* and the anostracan *T. platyurus* respectively, and a zebrafish embryo toxicity test. All three are freshwater species and were selected because of the usual disposal of olive oil mill effluents in surface waters.

The first two bioassays were performed according to the process of each protocol accompanying the test kit (Daphtoxkit F^{TM} pulex 2006; Thamnotoxkit F 2006).

The zebrafish embryo test was conducted according to the OECD (2004) and (DIN 38 415-T6 2001). The main benefits of using zebrafish as a toxicological model are their size, the optimum breeding and maintenance in laboratory conditions and their optical clarity which allows observation of different developmental stages. After fertilization, the eggs were collected and 4- to 8-cell stage eggs were selected for the test. Five concentrations of diluted effluent with artificial water and a control were used to fill the wells of 24-well dishes. Five eggs were placed into each well. Artificial water, which was also used as the control, was prepared with specific reagents described in OECD (2004). Incubation took place at 25°C for 48 h. The selected endpoints – coagulation, missing somites, missing tail detachment from yolk sac, missing spontaneous movement, missing eye formation, missing heartbeat – were considered lethal endpoints and were reported after 24 and 48 h.

In all toxicity tests, repetition was necessary in the case that the control group mortality exceeded 10%. Toxicity test results are expressed in LC_{50}-24 h values for the Thamnotoxkit *F* test and in LC_{50}-24 h & 48 h values for the Daphtoxkit F^{TM} *pulex* microbiotest and the zebrafish embryo test. Lethal concentrations were derived by the calculation of the concentration that provoked mortality to 50% of the test organisms, from the concentration-lethal curves for each sample.

SPSS statistical package (SPSS, 16.0) was used for all data analysis. As the parameters did not meet the Kolmogorov-Smirnoff test criteria for normality, Spearman correlation was used in order to identify probable correlations between data (values of all toxicity tests and the physicochemical parameters). Paired *t* test was performed between the results of the bioassays for each process in order to determine significance between the mean values.

Results and Discussion

All samples of the treated effluents, collected from the laboratory scale anaerobic reactor, were analyzed for several chemical and physical properties and mean values were obtained, presenting high fluctuation (Table 1). The composition of both OMW and OMSW is quite variable. Wide ranges in the main characteristics of such effluents have been reported (Paraskeva and Diamandopoulos 2006) and the composition of OMW varies substantially not only by region, but also by the type of process used, daily usage conditions, local operational procedures, olive type and maturity, soil composition of the olive groves, climatic conditions, the time between olive fruit gathering and olive oil extraction, the amount of water used, etc. (Niaounakis and Halvadakis 2006). As shown in Table 1, the phenolic content is significantly decreased after treatment as in raw samples concentrations may reach 25 g l^{-1} (McNamara et al. 2008), which may be partially due to the 25% dilution, whereas the remaining elements demonstrate high values.

In order to evaluate the environmental impact of treated effluents and consequently the integrated operation of such a reactor, toxicity test were conducted and the obtained LC_{50} values are shown in Table 2. For the treated OMW samples, the toxicity results of *T. platyurus* (24 h test) and zebrafish (24 and 48 h test) are quite similar, with a mean value of LC_{50} 1.77% and 1.99% and 1.52% respectively, opposed to *D. pulex* with a mean LC_{50} value of 4.38% for the 48 h toxicity tests. *D. pulex* seems less sensitive against the treated olive pulp samples, resulting to a similar 48 h LC_{50} average of 4.48%. Overall, the treated effluents indicate high levels of toxicity even at high dilutions.

The average values for all tests were transformed to toxic units (TU) based on the formula $TU = (1/LC_{50})*100$ (Isidori et al. 2000). All samples were classified as "very toxic" (TU = 11–100). Due to the high fluctuation in the LC_{50} values among the samples, it is interesting to comment the differences observed in Fig. 1. *D. pulex* seams to be a less sensitive organism than *T. platyurus* and *D. rerio*. 26% of the treated olive oil mill effluents are characterized as "extremely toxic" (TU > 100) by the 48 h zebrafish test, 8% by the Thamnotoxkit. The remaining 74% and 82% respectively of the rest are considered as "very toxic". On the other hand, 3 samples are non-toxic for the 24 h *D. pulex* test, whereas 18% of the samples are "toxic" (TU = 1–10) and 82% "very toxic". The exposure time progresses the toxicity effect, as the results of the 48 h test classify 5% of the samples in the "toxic" and 95% in the "very toxic" category.

Agro-industrial effluents are of great concern in countries with high primary production such as Greece, because they cause deleterious environmental effects. The complex composition, the great variability of OMW and the presence of inhibitory components (phenolics) coupled with the dispersal of the olive oil mill units, pose not only technical but also economical barriers for efficient effluent treatment

Table 1 Composition of treated OMW and OMSW samples

	Average OMW	Range OMW	Average OMSW	Range OMSW
NO_3^- (mg/l)	301.3	69.7–898.1	321.8	58.5–777.3
NO^- (mg/l)	10.0	0.6–43.2	5.9	0.6–18.3
NH_3–N (mg/l)	89.6	1.3–777.1	81.0	2.2–276.0
SO_4^{2-} (mg/l)	881.0	22.0–4576.0	352.5	23.7–1560.0
PO_4^{3-} (mg/l)	1548.9	29.1–6903.4	1126.3	387.1–3238.2
Cl^- (mg/l)	106.5	6.3–496.5	36.6	5.0–104.5
TDS (g/l)	4.8	1.1–15.8	4.2	1.9–17.8
Phenols (g/l)	1.4	0.5–3.4	1.4	0.3–4.9
Tannin (g/l)	0.84	0.03–2.01	0.75	0.14–1.97
Lignin (g/l)	3.05	0.11–7.31	2.73	0.52–7.14
COD (g/l)	19.04	17.05–21.71	23.28	11.9–32.8
pH	5.0	4.8–6.4	4.9	4.7–5.2
Conductivity (mS/cm)	7.3	1.4–25.7	5.1	3.7–8.8

Table 2 LC_{50}* values of treated effluents for all acute toxicity tests

Sample	Daphtoxkit		Thamnotoxkit	Zebrafish	
	LC_{50} 24 h	LC_{50} 48 h	LC_{50} 24 h	LC_{50} 24 h	LC_{50} 48 h
OMW average	7.64	4.38	1.77	1.99	1.52
SD	4.5	2.6	0.8	1.1	0.8
OMSW average	8.83	4.48	2.32	2.09	1.63
SD	6.5	2.9	1.0	0.7	0.8

* %, v/v in artificial water

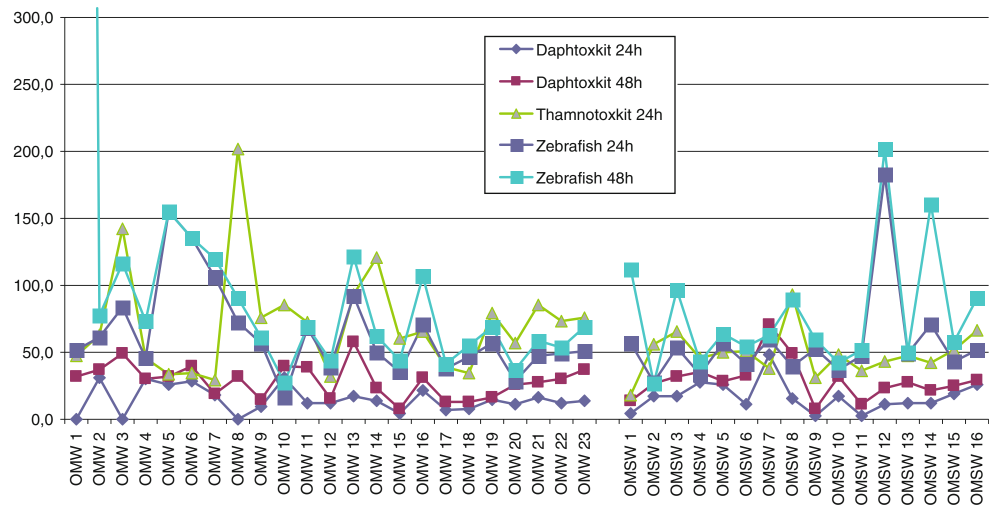

Fig. 1 Toxic units (*TU*) of the treated samples, resulted from all tests

Table 3 Spearman correlation coefficient between physicochemical parameters of treated OMW and OMSW effluents and the toxicity test results

	NO_3^-	NO_2^-	NH_3N	SO_4^{2-}	PO_4^{3-}	Cl^-	Phenols	COD
Treated OMW								
Daphtoxkit LC_{50} 24 h	−0.534**	−0.560**	−0.359*	−0.525**	−0.485**	−0.604**		−0.416*
Daphtoxkit LC_{50} 48 h	−0.721**	−0.530**				−0.628**		−0.624**
Thamnotoxkit LC_{50} 24 h							−0.485*	
Zebrafish test LC_{50} 24 h						−0.451*		
Zebrafish test LC_{50} 48 h						−0.433*		
Treated OMSW								
Daphtoxkit LC_{50} 24 h	−0.579*	−0.612*	−0.553*	−0.644**	−0.515*	−0.641**		
Daphtoxkit LC_{50} 48 h	−0.600*		−0.624*	−0.538*		−0.559*		−0.726**
Thamnotoxkit LC_{50} 24 h								−0.624**

* $p < 0.05$, ** $p < 0.01$

(McNamara et al. 2008). A number of treatment processes have been proposed by many researchers (McNamara et al. 2008; Niaounakis and Halvadakis 2006; Paraskeva and Diamandopoulos 2006), but biological processes provide some of the most viable options and anaerobic treatment seems to be recognized as an effective method due to the reduction of the high organic load and the production of a usable biogas fuel.

Figure 1 demonstrates the sensitivity of all organisms against the treated samples, as amplified by the correlations with the chemical parameters by both effluent groups (Table 3). The Daphtoxkit results are influenced by the most parameters, but show no correlation to phenols. This may be an indication of why *Daphnia pulex* had lower mortality percentages in relation to the other two organisms. *Thamnocephalus platyurus* and *Danio rerio*, though quite sensitive against the treated effluents, demonstrated correlation only to phenols – COD and total chloride respectively. This may imply an effect by other constituents that were not analyzed in this study. COD has shown significant correlation with the raw effluent (Rouvalis et al. 2004) but in this study, due to the lower concentration values, only *Daphnia pulex* seems to be influenced by the organic load.

Previous studies have determined the toxicity of raw OMW (Rouvalis et al. 2004), that were classified as "toxic" to "very toxic". The purpose of this project was to evaluate the potential of safe effluent discharge after anaerobic treatment. The outflow of the reactor was characterized as "very toxic" against the tested organisms for both types of processing systems. Paired *t* test between the bioassays for each process showed no significant differences between the mean values. Thus the outflow of a three-phase and a two-phase process did not demonstrate differences in terms of the toxicity effect to freshwater organisms. The replacement of three-phase olive mills by their two-phase counterparts may have advantages in terms of wastewater volume reduction but, according to this study, the environmental effect of the residue must be considered even after treatment.

Anaerobic treatment is known to significantly reduce the organic load of wastes by a percentage of 70%–89% (McNamara et al. 2008). In this case, treatment resulted to reduced COD concentration but all other chemical parameters analyzed remained high, above the acceptable limits prescribed by the Greek government for disposal in aquatic environments.

Parameters such as SO_4^{2-}, NO_2^-, NO_3^-, NH_3N and COD demonstrated correlations to the test results of the *D. pulex* bioassay (Table 3) and possibly contribute to the toxic effect of the treated effluent. This is in accordance to other studies where it has been proven that such elements have a toxic effect on freshwater species (Camargo et al. 2005). In the case of Cl^-, Postma et al. (2002) has shown that it is toxic for freshwater organisms such as *D. magna* and *Branchiodanio rerio* ($LC_{50} = 1.2$–7.6 g/L) at concentrations higher than those found in the analyzed samples of the present study. But it has been noted that Cl^- concentrations influence the results of the toxicity tests (Postma et al. 2002), a fact that can be evident from the correlation factors (Table 3) in the case of the Daphtoxkit and zebrafish test.

Consequently, the overall treatment as a most promising method to produce hydrogen through anaerobic fermentation of low cost substrates such as olive oil mill wastewaters (Benemann 1996) must be followed by another treatment for improving the quality of the final effluent, permitting the safe discharge to the environment. Thus, toxicity evaluation is a prerequisite in waste characterization. The data derived from this work can be useful for the management and protection of aquatic environments through risk assessment assays, as toxicological databases are scarce. In this study bioassays, in the form of toxkits and embryo tests, proved reliable for toxicity screening of wastes and specifically for such complex effluents.

Finally, hydrogen production through anaerobic treatment of the olive oil mill by-products is of great interest, but cannot be considered as the ultimate method in terms of environmentally friendly effluents. A combination with other suitable treatment methods which will deduct nutrients and phenolics could be further investigated and possibly lead to environmentally acceptable results.

Acknowledgments We wish to thank Dr. Eleni Koutrouli and professor Gerasimos Lyberatos, University of Patras, Department of Chemical Engineering, Laboratory of Biochemical Engineering and Environmental Technology, Greece. This work could not have been completed without the collaboration with Dr. Koutrouli who conducted the effluent treatment in the anaerobic fermentation system.

References

APHA, AWWA, WPCF (1989) Standard methods for the examination of water and wastewater, 17th edn. American Public Health Association, Washington DC, USA

Aranda E, García-Romera I, Ocampo JA, Carbone V, Malorni A, Sannino F, De Martino A, Capasso R (2007) Reusing ethyl acetate and aqueous exhausted fractions of dry olive mill residue by saprobe fungi. Chemosphere 66:67–74

Benemann J (1996) Hydrogen biotechnology: progress and prospects. Nat Biotechnol 14(9):1101–1103

Box JD (1983) Investigation of the Folin-Ciocalteau phenol reagent for the determination of polyphenolic substances in natural waters. Water Res 17:511–525

Camargo JA, Alonso A, Salamanca A (2005) Nitrate toxicity to aquatic animals: a review with new data for freshwater invertebrates. Chemosphere 58:1255–1267

DIN 38 415-T6 (2001) Bestimmung der nicht akut giftigen Wirkung von Abwasser auf die Entwicklung von Fischeiern über Verdünnungsstufen (T6). Deutsche Einheitsverfahren zur Wasser-Abwasser und Schlammuntersuchung

Eroğlu E, Gündüz U, Yücel M, Türker L, Eroğlu I (2004) Photobiological hydrogen production by using olive mill wastewater as a sole substrate source. Int J Hydrogen Energ 29:163–171

Isidori M, Parrella A, Piazza CML, Strada R (2000) Toxicity screening of surface waters in southern Italy with toxkit microbiotests. In: Persoone G, Janssen CR, De Coen W (eds) New microbiotests for routine toxicity screening and biomonitoring. Kluwer Academic/Plenum Publishers, New York, pp 289–293

Kapellakis IE, Tsagarakis KP, Crowther JC (2008) Olive oil history, production and by-product management. Rev Environ Sci Biotechnol 7:1–26

Koutrouli EC, Gavala HN, Skiadas IV, Lyberatos G (2006) Mesophilic biohydrogen production from olive pulp. Process Saf Environ Prot 84(B4):285–289

McNamara CJ, Anastasiou CC, O'Flaherty V, Mitchel R (2008) Review bioremediation of olive mill wastewater. Int Biodeter Biodegr 61:127–134

Mekki A, Dhouib A, Feki F, Sayadi S (2008) Assessment of toxicity of the untreated and treated olive mill wastewaters and soil irrigated by using microbiotests. Ecotox Environ Saf 69:488–495

Niaounakis M, Halvadakis CP (2006) Olive processing waste management: literature review and patent survey, 2nd edn. Elsevier, Amsterdam

OECD Guideline for testing of chemicals (2004) Fish embryo toxicity test with the zebrafish *Danio rerio*

Paraskeva P, Diamandopoulos E (2006) Technologies for olive mill wastewater (OMW) treatment: a review. J Chem Technol Biot 81:1475–1485

Postma JF, De Valk S, Dubbeldam M, Maas JL, Tonkes M, Schipper CA, Kater BJ (2002) Confounding factors in bioassays with freshwater and marine organisms. Ecotox Environ Saf 53(2):226–237

Daphtoxkit F^{TM} pulex (2006) Crustacean toxicity screening test for freshwater, Standard Operational Procedure. MicroBioTests, Nazareth, Belgium

Rouvalis A, Iliopoulou–Georgudaki J, Lyberatos G (2004) Application of two microbiotests for acute toxicity evaluation of olive mill wastewaters. Fresenius Environ Bull 13(5):458–464

Sayadi S, Allouche N, Jaoua M, Aloui F (2000) Detrimental effects of high molecular-mass polyphenols on olive mill wastewater biotreatment. Process Biochem 35:725–735

Thamnotoxkit F^{TM} (2006) Crustacean toxicity screening test for freshwater, Standard Operational Procedure. MicroBioTests, Nazareth, Belgium