Applied Soil Ecology 119 (2017) 18–25

Contents lists available at ScienceDirect

Applied Soil Ecology

journal homepage: www.elsevier.com/locate/apsoil

Assessment of toxicological effects of raw and bioremediated olive mill waste in the earthworm *Eisenia fetida*: A biomarker approach for sustainable agriculture

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ARTICLE INFO

Keywords:
Olive mill waste
Polyphenols
Biomarkers
Earthworms
Bioremediation

ABSTRACT

The olive oil extraction process produces large amounts of olive mill waste (OMW) that can be re-used in agriculture by land spreading. OMW has a low pH, high salinity and high levels of polyphenols and is potentially dangerous for the environment. It is therefore mandatory to develop procedures able to produce OMW with lower environmental impact. The first aim of the present study was to evaluate the toxicological effects of two different typologies of OMW: raw Two-Phase Olive Mill Waste (TPOMW) and raw Olive Mill Waste Waters (OMWW), on a soil bioindicator, the earthworm *Eisenia fetida,* using neurotoxicity (AChE), oxidative stress (LPO, CAT) and genotoxicity (comet assay) biomarkers. The second aim was to test the efficacy of a composting/bioaugmentation process by investigating the toxicological effects of the bioremediated OMW. *E. fetida* was exposed for 72 h to increasing concentrations (12.5, 25, 50%) of raw and bioremediated TPOMW and OMWW in an artificial soil (50% potting soil + 50% quartz sand). Raw OMW caused earthworm mortality at high doses and measurable biochemical and cellular effects at lower doses. The main cause of these effects is probably the high level of polyphenols present in the OMW. Neurotoxic effects, induction of oxidative stress and genotoxic effects were highlighted. The most evident toxicological effects were produced by the TPMW. The evaluation of the bioremediation process efficacy revealed that a high decrease in acidity and polyphenol content corresponds to a decrease of toxicological effects. This study contributed to assess the environmental sustainability of the bioremediated OMWs, to be used as fertilizers in agriculture.

1. Introduction

The olive oil industry is an important sector of the agro-industry in the European Union with a total olive oil production average higher than 2 million tons/year in the last ten years. Unfortunately, as a consequence, more than 30 million tons of olive mill waste (OMW) are generated each year. OMW presents high values of biological oxygen demand (BOD) (50–100 g l^{-1}) as well as of chemical oxygen demand (COD) (80–200 g l^{-1}), a slightly acidic pH, and high organic matter content (Kavvadias et al., 2010). Its lipid content is usually high and depends on the extraction yield. OMW is composed by 50–94% of water, 4–16% of organic compounds, and 0.4–2.5% of mineral salts. The organic fraction contains 2–15% of phenolic components, such as caffeic acid, tyrosol, tannins, and anthocyanins (Hachicha et al., 2009).

The most widespread used system of oil extraction is the three-phase system, which produces olive oil, Olive Mill Waste Waters (OMWW) and dried pomace. To minimize the volume of waters used, and therefore reduce phenol washing, a more efficient extraction process was developed in the 1990s, the two-phase extraction process. Using this technology, the olive paste is separated into two phases: olive oil and the Two-Phase Olive Mill Waste (TPOMW). TPOMW has strong odor and semi-solid texture that makes its manipulation and transport difficult.

By-products derived by oil extraction represent a serious environmental problem, mainly because of their high organic load (Roig et al., 2006), lipids, organic acids and polyphenols content. They can also contain traces of pesticides used in olive cultivation. More than 50 different phenolic compounds have been identified in OMWW (Torrecilla, 2010) until now. Oleuropein, an ester of hydroxytyrosol and the elenolic acid glucoside, is the most abundant polyphenol present in many varieties of olives (Montedoro et al., 1993). It is found in the humid pomace and vegetation water at high concentrations because

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http://dx.doi.org/10.1016/j.apsoil.2017.05.016
Received 19 January 2017; Received in revised form 17 May 2017; Accepted 22 May 2017
Available online 06 June 2017

of its high hydrophilicity (the partition coefficient of oil/water is 0.010). Polyphenols seem to be responsible for the phytotoxic and antimicrobial effect of by-products and are potentially dangerous for the environment and human health (Torrecilla, 2010). Studies conducted on the germination of *Cucumis sativus* seeds, *Lepidium sativum, Sorghum bicolor* and on the mortality of animals like *Daphnia magna, Thamnocephaus platyurus* and *Brachionus calyciflorus*, showed a high toxicity of tyrosol and hydroxytyrosol catechol polyphenols (Fiorentino et al., 2004; Isidori et al., 2005; Paixão et al., 1999). Different studies have established that several types of plant polyphenols, either alone (Yen et al., 2002) or in the presence of copper ions, can cause oxidative strand breakage in DNA. Bhat et al. (2007) demonstrated genotoxicity of increasing concentrations of gallic acid, caffeic acid and its derivates to human lymphocytes.

Italian legislation permits the re-use of olive oil by-products in agriculture and they can be spread on the bare soil at controlled concentrations (50–80 tons/ha/year depending on the origin of OMW) before annual herbaceous crops planting or seeding (Aliakbarian et al., 2011) or in the olive groves. The disposal of these by-products represents one of the most expensive cost-effective problems that olive mill industries have to face (Dermeche et al., 2013).

The phytotoxic effects of OMW were investigated by several authors (Andreozzi et al., 2008; Barbera et al., 2014; Hanafi et al., 2011). Previous studies (Kavvadias et al., 2010; Magdich et al., 2012) demonstrated that olive mill waste has toxic effects on soil microorganisms due to high levels of polyphenols. The presence of micronuclei was observed in specimens of *Vibia faba* exposed to 10% OMWW (El Hajjouji et al., 2007) and in human cell cultures exposed to olive mill waste at an early stage of composting (Chowdhury et al., 2015). To our knowledge, no information is available about the toxicity of olive mill waste to soil invertebrates, although this is the main disposal target compartment. Danellakis et al. (2011) investigated the impact of OMWW as marine environment pollutant by evaluating a set of biomarkers in the mussel *Mytilus galloprovincialis*: neutral red retention (NRR), acetylcholinesterase (AChE) activity, lipid peroxidation (LPO), micronuclei frequency and comet assay. The results showed that OMW disposal into the marine environment could induce pre-pathological alterations in marine organisms (Danellakis et al., 2011).

There is a clear need for the development of more effective methodologies in order to reduce the toxicological potential of OMW before its re-use in agriculture. A promising one is the bioremediation process, typically monitored by measuring the concentrations of tracer contaminants (Wilson and Jones, 1993). However, because of the process complexity the chemical analysis alone does not give a complete view of its effectiveness. It is therefore necessary to integrate the chemical analysis with the evaluation of toxicological effects through the use of a set of biomarkers on test organisms (Płaza et al., 2005; Saterbak et al., 2000). Several studies showed the application of ecotoxicological tests, single or combined, in order to evaluate the efficiency of different techniques of soils, sediments and complex matrices bioremediation (da Costa et al., 2014; Maier et al., 2016), however no information is available on the use of this approach to test the bioremediation process of OMW.

In the "Rational management of human and natural resources in modern types of olive groves and of by-products of olive processing (Modolivi)" project, supported by Tuscany Region (Italy) under the EU Rural Development Programme (2007–2013), a bioremediation treatment was developed for two typologies of OMWs, with the aim to produce environmentally sustainable soil fertilizers. Raw OMWW and TPOMW were collected from Tuscan olive mills and subjected to a bioremediation process based on bioaugmentation. Fungi strains able to metabolize polyphenols were isolated from the two matrices, cultured and added to olive humid husks or olive mill wastewater, composted with chopped straw and olive leaves. Polyphenol concentrations and acidity were found to be strongly reduced at the end of the treatment (Parrotta et al., 2016).

The first aim of the present study, which is part of the Modolivi project, was to evaluate the toxicological effects of raw TPOMW and OMWW on a soil bioindicator, the earthworm *Eisenia fetida* (Savigny 1826), by using a set of biomarkers. Acetylcholinesterase (AChE) activity was measured to test potential neurotoxic effects, catalase (CAT) and lipid peroxidation (LPO) were tested to investigate oxidative stress and comet assay to assess genotoxic effects. *E. fetida* is a compost worm that feeds on organic matter in the top soil layer and it is widely used as a bioindicator to evaluate the toxicological effect of soil contaminants. Nowadays there are several published studies that evaluated biomarker responses in *Eisenia* spp. exposed to metals, organic contaminants and pesticides (Sforzini et al., 2015, 2012, 2011; Stepić et al., 2013; Velki and Hackenberger, 2013a, 2013b; Zhang et al., 2017). The second aim of our study was to test the effectiveness of the composting/bioaugmentation process by investigating the toxicological effects of bioremediated TPOMW and OMWW (bioTPOMW and bioOMWW) using the same biomarker approach applied to investigate the toxicity of raw OMW.

2. Materials and methods

2.1. Experimental matrices

Semi-solid waste derived from olive oil production in a two-phase olive mill (rawTPOMW) and waste water derived from a three-phase olive mill (rawOMWW) were collected in south Tuscany and subjected to a bioremediation process based on composting and bioaugmentation. The biorecovery process is explained in detail in the work of Parrotta et al. (2016). Briefly, fungi strains able to metabolize polyphenols were isolated from raw TPOMW and OMWW, grown and added to olive humid husks or olive mill wastewater, composted with chopped straw and olive leaves. Polyphenol concentration and pH were measured in both composted OMW before and during the bioremediation process, which lasted 6 months. As reported by Parrotta et al. (2016), levels of polyphenols in raw TPOMW were about 390 mg/kg and pH was 4, while levels of polyphenols in bioremediated TPOMW (bioTPOMW) decreased to about 25 mg/kg and pH increased to 6. Levels of polyphenols in raw OMWW were about 415 mg/kg and pH was 3, while levels of polyphenols in bioremediated OMWW (bioOMWW) decreased to about 290 mg/kg and pH increased to 5.

Matrices used for exposure of *E. fetida* were rawTPOMW, rawOMWW, bioTPOMW and bioOMWW.

2.2. Experimental design

2.2.1. Treatment with rawTPOMW and rawOMWW

The experiments were conducted using artificial soil prepared with 50% w/w organic potting soil and 50% w/w air-dried quartz sand. In the first phase of the study groups of 20 adult (0.18 g $\pm$ 0.06) worms (*E. fetida)* with well-developed clitellum purchased from Lombricoltura Compagnoni (Mandello sul Lario, Como, Italy) were kept in 1000 g of artificial soil placed in glass test containers. They were acclimatized for one week and subsequently exposed for 72 h under laboratory conditions at three different concentrations (12.5, 25 and 50% w/w) of raw TPOMW plus a positive (dimethoate) and a negative control. In a parallel experiment, bioindicators were exposed to three different concentration (12.5, 25 and 50% w/w) of raw OMWW plus a negative control. Dimethoate was suspended in distilled water and mixed with 1000 g soil to obtain a concentration of 0.6 mg/kg dry soil. Dimethoate is a widely used organophosphate insecticide used also in treatment of olive fruit flyer. The negative control groups were kept in untreated soil. All soils were moistened with deionized water to obtain the same humidity in all the matrix tested. The total moisture content was approximately 35% of the final wet weight and the test containers were maintained in a controlled chamber with a temperature of 20 $\pm$ 1 °C. The test was performed under controlled light–dark cycles (16 h light,