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Toxicity evaluation of olive oil mill wastewater and its polar fraction using multiple whole-organism bioassays

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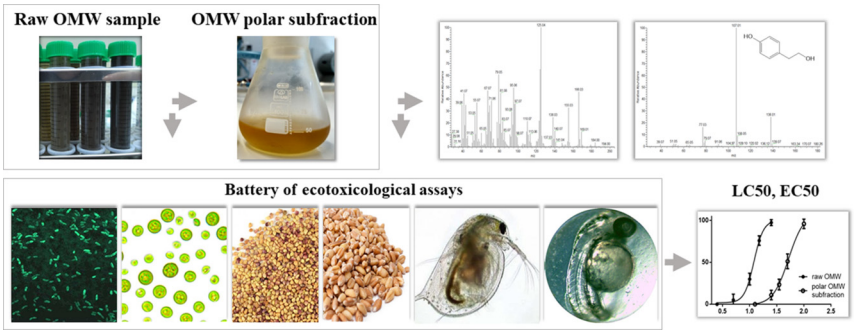
HIGHLIGHTS

- Raw OMW and polar fraction exhibited the highest toxicity on the photobacterium *Vibrio fischeri*.
- Tyrosol was the most common phenolic compound in the tested OMW.
- Multi-biomarker approach showed as a good indicator of OMW toxic potential.

GRAPHICAL ABSTRACT



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ABSTRACT

Olive mill wastewater (OMW) as a by-product of olive oil extraction process has significant polluting properties mainly related to high organic load, increased COD/BOD ratio, high phenolic content and relatively acidic pH. Raw OMW from Slovenian Istria olive oil mill and its polar fraction were investigated in this study. Chemical characterization of OMW polar fraction identified tyrosol as the most abundant phenolic product, followed by catechol. Lethal and sub-lethal effects of OMW matrix and its polar fraction were tested using a battery of bioassays with model organisms: bacteria *Vibrio fischeri*, algae *Chlorella vulgaris*, water fleas *Daphnia magna*, zebrafish *Danio rerio* embryos, clover *Trifolium repens* and wheat *Triticum aestivum*. Raw OMW sample was the most toxic to *V. fischeri* (EC_{50} = 0.24% of OMW sample final concentration), followed by *D. magna* (EC_{50} = 1.43%), *C. vulgaris* (EC_{50} = 5.20%), *D. rerio* (EC_{50} = 7.05%), seeds *T. repens* (EC_{50} = 8.68%) and *T. aestivum* (EC_{50} = 11.58%). Similar toxicity trend was observed during exposure to OMW polar fraction, showing EC_{50} values 2.75–4.11 times lower comparing to raw OMW. Tested samples induced also sub-acute effects to clover and wheat (decreased roots, sprouts elongation); and to zebrafish embryos (increased mortality, higher abnormality rate, decreased hatching and

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pigmentation formation rate). A comprehensive approach using a battery of bioassays, like those used in this study should be applied during ecotoxicity monitoring of untreated and treated OMW.

1. Introduction

Olive processing and olive oil production are of great economic importance in different Mediterranean countries (e.g. Morocco, Spain, Italy, Tunisia, Greece, Turkey) and one of the fastest growing agro-food sectors in EU with >4% of average annual growth rate (Gómez-Caravaca et al., 2014; Xiong et al., 2014). Despite its economic importance, olive processing has been associated with several adverse effects on the environment causing resource depletion, land degradation, and waste production. The by-products derived from the olive oil extraction processes differ based on the used technologies and include olive oil (only 20% of the overall input volume), as well as two waste matrices known as olive husk (olive cake or olive pomace) and wastewater (Massoudinejad et al., 2014). Worldwide olive processing produces every year in a short period of time (i.e. between November and February) an amount of 7×10^6 up to 3×10^7 m^3 of olive mill wastewater (OMW). This quantity of OMW is equal to the pollution load of municipal sewage produced by 20–22 million people (Caporaso et al., 2018; Massoudinejad et al., 2014). OMW, as the main waste product, contains a high amount of organic material which disposal and management are of increasing environmental concern (Chatzistathis and Koutsos, 2017; Kavvadias et al., 2010). Its pollution load is mainly related to OMW's high biological and chemical oxygen demand (BOD and COD), but also with a high content of phenolic compounds, organic and solid matter, metals, mineral substances (i.e. potassium, phosphorous, calcium) and acidic character with pH values between 3.0 and 5.9 characterizing OMWs as an overall recalcitrant effluent (Chatzistathis and Koutsos, 2017; Lozano-García et al., 2011; Magdich et al., 2013). Besides its strong organic content (BOD 35–110 g L^{-1}, COD 45–170 g L^{-1}) OMW contains high concentrations of recalcitrant compounds such as lignins and tannins which give it a characteristic dark color [52,270–180,000 mg/L Pt—Co units (Paraskeva and Diamadopoulos, 2006)]. Typical OMW composition by weight is 83–94% water, 4–16% organic compounds (of which 2–15% phenolic compounds) and 0.4–2.5% mineral salts (Davies et al., 2004). Only 1–2% of the total phenolic compounds of the olive fruit remains in the oil phase, while approximately 53% is lost either in the OMW or in the olive husk (approximately 45%), which is in agreement with the hydrophilic nature of phenolics and their high solubility in the water phase (Caporaso et al., 2018; McNamara et al., 2008; Rodis et al., 2002). Concentration of phenols in OMW varies from 1 to >3000 mg mL^{-1} (Allouche et al., 2004; Justino et al., 2012; Roig et al., 2006). Main phenolic compounds identified in OMW include cinnamic acid derivatives (e.g. caffeic, coumaric and ferulic acid), benzoic acid derivatives (e.g. protocatechuic, hydrobenzoic, vanillic and gallic acid) and tyrosol derivatives such as p- hydroxytyrosol and 4-hydroxyphenylacetic acid (Justino et al., 2012). Olive phenols possess a diverse range of bioactivities such as antioxidant, antimicrobial, radical scavenger activities, uncoupling agent's role, anti-carcinogenic and metal-chelating properties (Gómez-Caravaca et al., 2014; Justino et al., 2012). Although phenolic compounds may have beneficial effects for humans, they conversely contribute to the high toxicity of OMWs and their potential environmental impact (Pardo et al., 2017).

Among available EU directives, the Waste Framework Directive Directive (2008) includes rules on hazardous waste and waste oils, while liquid wastes from olive oil production fall under the Urban Waste Water Treatment Directive (Directive, 1991), which also regulates treatment and discharge of wastewater from the olive oil industry sector (Komnitsas and Zaharaki, 2012). Despite the fact that no dedicated EU legislation exists on OMW management, each EU production country has implemented its own national guidelines. Due to the lack of strict regulations, the OMWs are still often being discharged into sewer systems and different environmental compartments such as freshwaters and soils (Caporaso et al., 2018; Kavvadias et al., 2010). Considering that the approximated amount of 1.5 million tons of untreated OMWs are annually disposed in a short period, it is obvious that OMWs are becoming a potential environmental issue due to their pollution load and effects on the quality of soil, surface and groundwater as well as freshwater and marine organisms (Karaouzas et al., 2011; Mekki et al., 2008; Pavlidou et al., 2014). However, controversial results have been reported concerning the effects of OMW on the environment. While some authors found a beneficial role of OMW on soil quality and suggested OMW as an inexpensive source of nutrients that can replace chemical fertilizers and restore degraded ecosystems (Belaqziz et al., 2016; Vella et al., 2016), others emphasized negative impact on soil properties/quality and subsequently increased groundwater contamination (Kavvadias et al., 2010; López-Piñeiro et al., 2011). Various treatment methods (e.g. aerobic, anaerobic digestion, physiochemical and biological) have been proposed until now, but there are usually too expensive for most olive-oil mills (Chatzistathis and Koutsos, 2017; Ioannou-Ttofa et al., 2017). In addition, studies integrated with multiple ecotoxicological tests for a relevant assessment of its effectiveness in term of toxicity reduction before and after such treatment are scarce (Justino et al., 2012).

Although available ecotoxicological studies on OMW are based on the evaluation of lethal and sublethal endpoints using bioassays with different groups of organisms (i.e. plant species, bacteria, crustaceans), they are very difficult to compare due to different experimental design, exposure times, applied OMW dilutions and selected evaluated endpoints (Table 1). These facts emphasize the need for the determination of critical OMW parameters and phenolic components that contribute mainly to OMW's toxic potential.

The aim of the present work was to study the impact of olive oil wastes on a battery of model organisms (on various levels of biological organization, different trophic level and with different sensitivities) to assess the toxic potential of raw OMW and its polar fraction. Specifically, the objectives of this study include: *i*) chemical characterization of OMW; *ii*) identification of the OMW components among phenolic group which are directly correlated with OMWs toxicity; *iii*) evaluation of sub-lethal effects and toxic potential of OMW and its polar fraction on bacteria *Vibrio fischeri* (ISO, 2007), algae *Chlorella vulgaris* (OECD, 2011), microcrustacean *Dapnia magna* (OECD, 2004), fish *Danio rerio* (OECD, 2013) and plants *Trifolium repens* and *Triticum aestivum*.

By applying a battery of bioassays it could be possible to evaluate the ecotoxicity potential of OMW on different compartments (i.e. soil and water) as well as on their biota and estimate the efficiency of up-scale methodologies considered for OMW treatment and reduction of its toxicity.

2. Materials and methods

2.1. Chemicals

Acetonitrile (LC-MS grade) was obtained from Fisher Chemical (Fair Lawn, New Jersey), while methanol (CAS No. 67-56-1), dichloromethane (CAS No. 75-09-2) and formic acid (CAS No. 64-18-6) were purchased from Sigma-Aldrich (Deisenhofen, Germany). Artificial/reconstituted water and BBM were prepared using chemicals all purchased from Sigma Aldrich (Deisenhofen, Germany), in accordance with standardized protocols: ISO (1996), OECD (2004) and OECD

Table 1
A selection of most relevant toxicity data on model organisms exposed to olive oil mill wastewater.

Location site	Model organism	Exposure mode	Duration	Endpoint	Effects	Ref.
Achaia, Greece	*Daphnia pulex*	Serial dilutions of the raw OMW and OMW treated with *Pleurotus ostreatus*	48 h	Acute toxicity	LC50 = 1.27% of raw OMW LC50 = 1.09% of treated OMW	Rouvalis et al., 2013
	Danio rerio embryos				LC50 of raw OMW = 0.48% LC50 = 1.10% of treated OMW	
	Thamnocephalus platyurus		24 h		LC50 OMW = 0.96% of raw LC50 = 1.91% of treated OMW	
Messinia, Greece	Palaemonidae shrimp	OMW diluted with river water to initiate concentrations of 0.2%, 0.4%, 1.0% and 2.0%	24 h		LC50 = 0.7%	Pavlidou et al., 2014
İznik, Turkey	*Helianthus annuus*	Dilutions (1/1,1/10,1/100) of raw OMW applied as irrigation water	72, 120, 240 h	Genotoxicity	Significant damage in DNA's nucleotide and genomic structure	Aybeke, 2018
Tuscany, Italy	*Eisenia fetida*	12.5, 25, 50% of raw and bioremediated two-phase OMW and OMW in an artificial soil (50% potting soil +50% quartz sand)	72 h	Mortality, neurotoxicity, oxidative stress and genotoxicity	High mortality; inhibition of AchE activity; significant oxidative stress; DNA damage	Campani et al., 2017
Cordoba, Argentina	*Lactuca sativa*	Seeds and seedlings exposed to a range of OMW dilutions	48 and 120 h	Phytotoxicity	Inhibitory effects on seed germination (LC50 = 645.7 μg $GAEL^{-1}$ PAE) and seedling growth	Pierantozzi et al., 2012
	Artemia salina *Allium cepa*	Range of OMW dilutions	24 h	Acute toxicity Genotoxicity	High mortality; LC50 = 166.0 μg $GAEL^{-1}$ PAE Mitotic inhibition	
	Salmonella thyphimurium		48 h		No mutagenic activity in TA98 and TA100 strains	
Near the village of Murça, Portugal	*Lepidium sativum*	Three dilutions of raw OMW and OMW treated with the *Candida oleophila* [50, 8.5 and 4.2% (v/v)]	120 h	Phytotoxicity	Very high inhibitory effects on seed germination and root elongation	Amaral et al., 2012
	Vibrio fischeri	Dilutions of raw OMW and OMW treated with the *Candida oleophila*	15 min	Bioluminescence	LC50 = 3.30% of raw OMW LC50 = 5.50% of treated OMW	
Patras, Greece	*Mytilus galloprovincialis*	Raw OMW diluted with artificial seawater to obtain dilutions (1, 0.2, 0.1, 0.01% v/v)	96 h for mortality assay, 5 days for other endpoints	Acute toxicity, nuclear abnormalities, AChE activity, oxidative stress, genotoxicity	Mortality at concentrations higher than 1 and 0.2%; higher nuclear abnormalities; inhibition of AChE activity; an increase of malondialdehyde levels; increase in levels of DNA damage	Danellakis et al., 2011
Iran	Radish seeds Cress seeds Tomato seeds	Raw and diluted OMW (T1 = 0.1%, T2 = 1%, T3 = 10%, T4 = 25%, T5 = 50%, T6 = 100% OMW)	5 days	Phytotoxicity	Raw OMW at concentrations higher then 25% OMW blocked seed germination	Massoudinejad et al., 2014
Near the city of Kalamata, Southern Greece	*Spinacea oleracea*	One-month-old plants subjected to OMW (1:10 OMW/Hoagland and 1:20 OMW/Hoagland)	1 month	Phytotoxicity and chemical analysis	Suppressed seed germination; reduction of biomass production; reduction in the root/shoot ratio; loss of the photosynthetic pigments; overaccumulation of total polyphenols; limited nutrient accumulation	Asfi et al., 2012
Evrotas River Basin, Southern Greece	Macroinvertebrates	8 sampling sites were selected along 4 streams that receive OMW	2 years	Macroinvertebrate community variation	During the OMW discharge period the number and abundance of taxa were significantly decreased	Karaouzas et al., 2011
Lake Iznik region, Turkey	*Pelophylax ridibundus*	Larvae exposed to 10, 25 and 50% of OMW with and without extracting the phenolic compounds	From 2 min to 3 days	Acute toxicity; behavioral change	Hyperactivity symptoms; loss of balance and remaining motionlessness	Inceli and Sengezer-Inceli, 2012

(2011). Potassium dichromate (CAS No. 7778-50-9; $K_2Cr_2O_7$), sodium chloride (CAS No. 7647-14-5; NaCl) were also obtained from Sigma-Aldrich (Deisenhofen, Germany).

2.2. Collection and physico-chemical analyses of OMW samples

Fresh OMW was obtained from olive oil production plant located in Slovenian Istria, which uses a traditional discontinuous press for the extraction of olive oil. This process generates a solid fraction (olive husk), an emulsion containing the olive oil and water phase (wastewater). The separation of olive oil and the remaining wastewater is achieved by vertical centrifugation or decantation (Souilem et al., 2017).

OMW samples were collected in January 2017 and stored at −20 °C. Prior to testing an initial chemical and physical determination of OMW main constituents was performed (Table 2). Total chemical oxygen (COD) demand was measured using open reflux method, while biochemical oxygen demand (BOD_5) was determined by a 5-day BOD test. Total suspended (TSS) and dissolved solids (TDS) were measured in accordance with the Standard Method for Analysis of Waters and Wastewaters (APHA, 1999). Tested OMW sample was filtered through a Whatman GF/C membrane filter where the filtrate was used to determine the TDS, while the particles on the filter paper (after drying to a constant weight at 100 °C) were used to determine the TSS (APHA, 1999). Conductivity, pH and salinity were measured using Multiparameter portable meter MultiLine® Multi 3620 IDS, set with TetraCon 925 electrode. Total organic (TOC) and inorganic (TIC) carbon, and total nitrogen (T_N) were analyzed photometrically (Spectroquant® Vega 400) using standard kits (Hach cuvette test 60–735 mg/LC and 20–100 mg/L TN_b, respectively). Total phenolic content was determined following the colorimetric method of Folin-Ciocalteau (Vitali Čepo et al., 2017).

For trace metal analysis OMW sample preparation is done by acidic digestion method. The sample (200 mg) is taken into a digestion vessel

Table 2
Physio-chemical properties of raw OMW.

a)

Physio-chemical properties	Values
pH	4.44 ± 0.03
Color	Brown
Opacity	High
Total suspended solids (TSS; g L^{-1})	26.35 ± 0.91
Total dissolved solids (TDS; g L^{-1})	30.99 ± 4.25
Chemical oxygen demand (COD; g L^{-1} O_2)	129.55 ± 3.82
Biochemical oxygen demand (BOD_5; g L^{-1} O_2)	41.21 ± 2.10
Total organic carbon (TOC; g L^{-1})	43.6 ± 1.20
Total inorganic carbon (TIC; g L^{-1})	1.06 ± 0.004
Total nitrogen (T_N; g L^{-1})	0.42 ± 0.04
Total phenolic compounds (g GAE[a] L^{-1})	9.22 ± 0.17
Conductivity (mS/cm)	19.37 ± 0.04
Salinity (%)	1.60 ± 0.004
Biodegradability (BOD_5/COD)	0.32 ± 0.01

b)

Metals (μg L^{-1})	Values
Iron	494.46 ± 3.44
Magnesium	467.88 ± 2.10
Zinc	25.53 ± 2.09
Manganese	8.22 ± 0.14
Copper	3.53 ± 0.21
Nickel	2.43 ± 0.47
Chromium	1.15 ± 0.15
Lead	0.11 ± 0.02
Cadmium	$63.34 \times 10^{-3} \pm 0.78 \times 10^{-3}$
Mercury	$32.23 \times 10^{-3} \pm 7.78 \times 10^{-3}$

[a] GAE – gallic acid equivalents.

and 3 mL of nitric acid and 1 mL of hydrogen peroxide are added and the mixture is digested in a microwave oven (Milestone) until a clear solution is obtained. The temperature of the oven is increased slowly at a gradual ramp of about 10 °C and maintained at 200 °C for about 20 min. The digested solution was mixed, transferred to 50-mL sample vial, added 1.25 mL hydrochloric acid and made up with deionized water for analysis by ICP-MS spectrometer (Agilent 7900). The precision and accuracy of the results were assessed by determining repeatability and recovery of the analysis of laboratory control sample, matrix spike and matrix spike duplicate samples. For doing so, each sample was spiked in replicates of six at near mid-range calibration concentration. The spiked sample was digested and analyzed following the same analytical procedure as the samples. All analyses were performed in duplicate.

2.3. Extraction of phenolic compounds from OMW

Polar fraction was obtained by solid-phase extraction (SPE) of raw OMW sample (De la Torre-Carbot et al., 2005). Five hundred mL of the sample was passed through DSC-18 cartridges (10 g; 20 mL; Supelco, UK) preconditioned with methanol (MeOH) and double distilled water (ddH_2O) mixture (1:2:1, v/v; ddH_2O: MeOH: ddH_2O). Columns were eluted with MeOH and acetonitrile (ACN) mixture (1:3; v/v). The eluates were pooled and evaporated to dryness under vacuum on a rotary evaporator (Büchi, Switzerland) under nitrogen flow at 26 °C. After evaporation, the dry residues were dissolved in 3 mL of double distilled water. Desired concentrations of polar fraction for further testing were prepared by additional diluting with appropriate medium prescribed for each bioassay (ISO, 2007; OECD, 2004, 2011, 2013). Polar fraction was divided into two portions, one for quantitative analysis of phenolic compounds by GC–MS and the other to determine the toxicity potential of OMW.

2.4. Chemical characterization of OMW by GC–MS

Analysis of phenolic compounds was carried out using both gas chromatography – mass spectrometry (GC–MS) with electron ionization (EI) and high resolution mass spectrometry (HRMS) with electrospray ionization (ESI). GC–MS analysis was performed with a triple quadrupole (QqQ) mass-spectrometer TSQ 8000 (Thermo Scientific, Bremen, Germany) coupled with a TRACE 1310 Gas Chromatograph (Thermo Scientific, Bremen, Germany). The EI source was kept at 250 °C, while the electron energy was 70 eV. Chromatographic separation was performed using capillary chromatographic column TraceGOLD™ TG-5SilMS (15 m length, internal diameter 250 μm and phase thickness 0.25 μm) with helium as a carrier-gas (1 mL min^{-1}). The column temperature was programmed as follows: 50 °C (2 min) – 10 °C/min – up to 280 °C (5 min); transfer line temperature – 320 °C. A volume of 1.0 μL of dichloromethane extract was injected into the heated injector at 270 °C in split ratio 1:10.

HRMS experiments were performed with Orbitrap Elite mass-spectrometer (Thermo Fisher Scientific GmbH, Bremen, Germany) with an ESI source. An aliquot of the initial extract (10.0 μL) was dissolved in 1.0 mL of ACN (Fisher Chemical, LC-MS grade). Formic acid (Sigma-Aldrich) was added to the ACN solution to carry on the analysis in positive mode. The sample was directly injected into the ion source. Sheath gas flow rate was at 10 arbitrary units, auxiliary and sweep gas flow rate was set to zero. The capillary temperature was set to 275 °C and the spray voltage to 3.5 kV. Accurate mass measurements were carried out in Orbitrap analyzer with 480,000 resolving power. The elemental composition of each fragment ion was calculated within 5 ppm mass accuracy. The spectra were recorded during 30 s. Both systems were controlled by the Xcalibur software, which was used for control, data acquisition and processing.

2.5. Ecotoxicity tests

Testing concentrations of raw OMW and polar fraction were prepared by diluting testing samples with the medium prescribed for each bioassay (ISO, 2007; OECD, 2004, 2011, 2013). Such toxicity testing of the raw OMW sample and their polar fraction was chosen in order to give insight into the potential carriers of toxicity of the samples.

Five different acute and sub-acute toxicity tests were conducted with raw OMW samples and their polar fraction: *i*) Bioluminescence test on marine bacteria *Vibrio fischeri* (ISO, 2007); *ii*) Algal growth inhibition test using freshwater algae *Chlorella vulgaris* (OECD, 2011); *iii*) Germination seed test using clover *Trifolium repens* and wheat *Triticum aestivum*; *iv*) *Daphnia magna* acute immobilization test (OECD, 2004); *v*) fish embryo toxicity test with zebrafish *Danio rerio* (OECD, 2013). Those model organisms were selected in order to determine the effects of OMW on various levels of biological organization, from bacteria, freshwater algae, plants, invertebrates and vertebrates.

In order to determine the concentration range of interest for further testing, preliminary experiments were performed for each model organism on a wide range of OMW percent concentration (1, 5, 10, 25, 50, 75 and 100%). Prior to the experiment, pH of the tested samples was adjusted in accordance with the OECD protocols (ISO, 2007; OECD, 2004, 2011, 2013). For germination seed test, pH was adjusted to 6.5 according to the observation of Deska et al. (2011). The schematic chart of all performed toxicity tests is shown in Fig. 1.

2.5.1. Bioluminescent bacteria test

Reduction of bioluminescence of the marine bacteria *V. fischeri* (Dr. Lange GmbH, Düsseldorf, Germany) following exposure to tested samples was determined according to (ISO, 2007). An aliquot (500 μL) of each dilution was mixed with 500 μL of the bacterial suspension. For positive control, 102 mg/L $K_2Cr_2O_7$ was used. For the preparation of serial dilutions and negative control, a 2% NaCl solution was used. The bioluminescence was measured after 30 min of incubation at 15 ± 1 °C

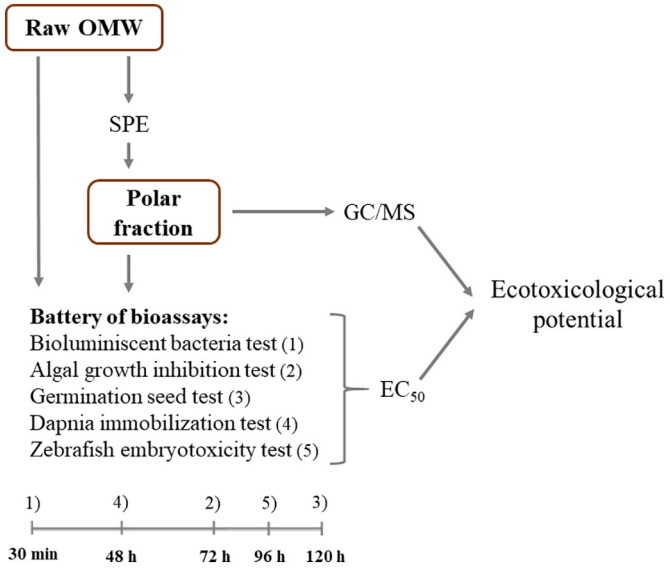

Fig. 1. Schematic flow chart of the experimental set-up. Legend: OMW – olive oil mill wastewater; SPE – solid phase extraction; GC/MS - gas chromatography – mass spectrometry; EC_{50} - concentration for which one half of the maximal effect is observed.

using a photomultiplier (LUMIStox 300 luminometer, Hach Dr. Lange GmbH, Germany), equipped with a thermo-block (LUMIStherm, Hach Dr. Lange GmbH, Germany). Regarding the brown color of the tested samples, color correction was done. The test was carried in two independent experiments (each with duplicates). Averages of bacteria luminescence due to the exposure were calculated and subsequently used for calculation of dilution on which 50% of bacteria luminescence inhibition was observed (EC_{50}).

2.5.2. Algal growth inhibition test

The toxicity towards the freshwater microalgae *C. vulgaris* (SAG 9.88, Collection of Algal Cultures, University of Göttingen, Germany) was determined following the OECD guideline (2011), with slight modifications. Sample dilutions were prepared with Bold Basal Medium (BBM), which was also used as a negative control. The algal inoculum was taken from an exponentially growing pre-culture and added into glass Erlenmeyer flasks containing 25 mL of the tested sample. The initial algae density was 10^5 cells per mL^{-1}. Each dilution was tested in three replicates. Algae were grown for 72 h at 25 ± 1 °C, under constant illumination of 7000 lx. The flasks were continuously shaken with an Innova 42R (New Brunswick) orbital thermo-shaker (160 rpm) to maintain algae suspensions homogeneous. The optical density of the algal cultures was read at 680 nm using Infinite M200 PRO (Tecan, Austria). Algae cell density was estimated from the calibration curve correlating absorbance values (680 nm) and cell density. Average of specific growth rates were calculated and subsequently used for calculation of dilution on which 50% of algae inhibition was observed (IC_{50}).

2.5.3. Germination seed test

Triticum sp. and *Trifolium* sp. are the most commonly occurring cultivar on agricultural soil and were selected for the phytotoxicity test. The germination assay was performed in three replicates with one monocotyledonous species, clover *T. repens* and one dicotyledon, wheat *T. aestivum*. The test was performed according to Visioli et al. (2014), with slight modifications. Uniform sized seeds were randomly placed on a culture dish (Sartsted Inc., USA) (10 seeds of clover, 8 seeds of wheat per dish) containing a filter paper moistened with 2.5 mL of each OMW sample. Petri dishes (100 × 15 mm) were covered and sealed with parafilm in order to prevent cross-contamination. Seeds were incubated at 24 ± 1 °C, in the dark. Controls were run in parallel on distilled water. After 5 days of incubation the germination percentage of seeds, as well as roots and sprouts elongation were measured. The length of 1 mm was used as the minimum length to be called roots/sprouts. Average of seed germination and sprout/root elongation were calculated and subsequently used for calculation of concentration on which 50% of seeds' germination was inhibited and of dilution on which sprout/root elongation was disabled for 50% comparing to control values (EC_{50}).

2.5.4. Daphnia acute immobilization test

Acute immobilization test was performed using water fleas *D. magna* (obtained from Biotechnical Faculty, University of Ljubljana), in accordance with OECD (2004). In brief, 15 mL of each test dilution was distributed in glass vials. Each dilution was tested in four replicates with six specimens, not older than 24 h. Vials were incubated at 20 ± 1 °C in the dark. Immobilization was recorded at 24 and 48 h of exposure. For negative control and dilution of tested samples was used reconstituted water (OECD, 2004). Average of *D. magna* immobilization after exposure was calculated and subsequently used for calculation of dilution on which 50% of specimens were immobilized (EC_{50}).

2.5.5. Zebrafish embryotoxicity test

Zebrafish maintenance and egg production have been described in detail in our previous study (Babić et al., 2017). ZET test was performed according to the OECD (2013), with slight modifications. Healthy mature wild type WIK strain zebrafish *D. rerio* were used for breeding [obtained from the European Zebrafish Resource Center of the Karlsruhe Institute of Technology (KIT)]. Fertilized eggs (up to 64-cell blastomeres) were added to 24-well plate (NEST Scientific, USA) containing 1 mL of aerated and pre-warmed (26 ± 1 °C) sample per well. 10 embryos per OMW dilution and controls were exposed in triplicates, containing a total of 30 embryos per tested sample. Plates were incubated (26 ± 1 °C) in the Innova 42 incubator shaker (New Brunswick, Canada) under regulated light/dark photoperiod (14/10). 30% of the test samples were replaced daily. Lethal and sub-lethal effects were estimated at 24, 48, 72 and 96 h of exposure using Leica DMIL LED inverted microscope (OECD, 2013), equipped with Leica EC3 digital camera. Averages of *D. rerio* mortality due to the exposure were calculated and subsequently used for calculation of dilution on which 50% of specimens were dead (LC_{50}). In addition, percentages of abnormalities, hatching and pigmentation formation were used for determination of dilution on which 50% of specimens were abnormal, did not hatch and/or were not pigmented completely (EC_{50}).

2.6. Statistical analysis

All tests were performed at least in triplicates. Results are presented as means ± SD. LC_{50} values with 95% confidence intervals were calculated by non-linear regression model using GraphPad Prism 6.01. (GraphPad Software Inc., USA).

3. Results

3.1. Physico-chemical properties of raw OMW

The physico-chemical parameters of all OMW samples and polar fraction are shown in Table 2 as mean values of each parameter obtained by duplicate samples of raw OMW. The inorganic content of OMW was mainly composed of metals. The metal content of OMW is shown in Table 2b. Iron and magnesium were the predominant metals (494.46 and 467.88 μg L^{-1}, respectively), followed in decreasing order by zinc (25.53 μg L^{-1}), copper, nickel and manganese.

3.2. Chemical characterization of polar fraction

Identification of organic compounds by GC–MS was done using mass spectral library database NIST14 and general fragmentation rules of

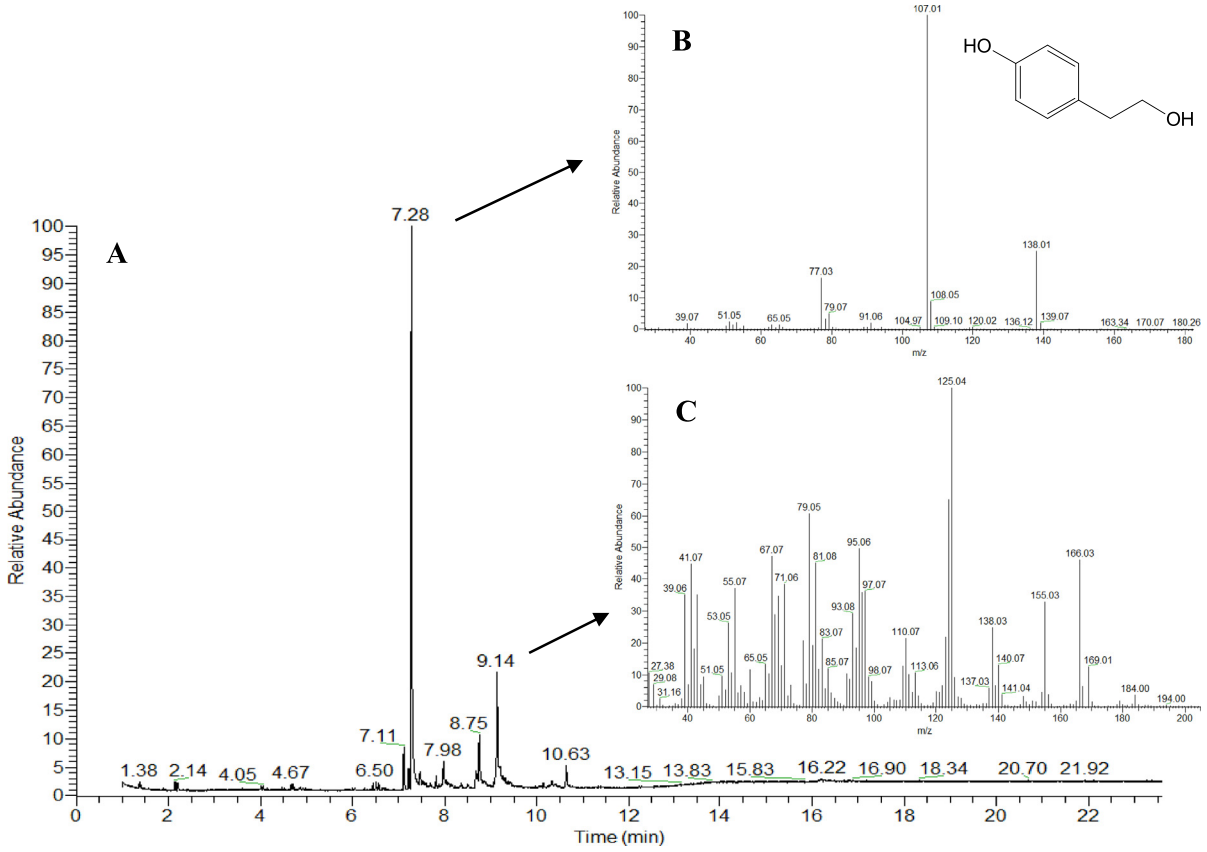

Fig. 2. (A) Total ion chromatogram (TIC) of the polar fraction (dichloromethane fraction of the extract). (B) EI mass spectrum of the compound with RT 7.28 min, identified by the library as 4-(2-hydroxyethyl)phenol. (C) EI mass spectrum of the compound with RT 9.14 min corresponding 2-methoxy-4-ethoxyresorcinol or 3-methoxy-4-ethoxycatechol.

organic compounds under electron ionization (McLafferty and Tureček, 1993). The analysis has shown a low amount of organic compounds transferred into the dichloromethane phase during extraction. The total ion chromatogram contains two intensive peaks (RT 7.28; RT 9.14) (Fig. 2A). According to the mass spectrum (Fig. 2B) the most abundant peak (RT 7.28 min) belongs to 4-(2-hydroxyethyl)phenol. This

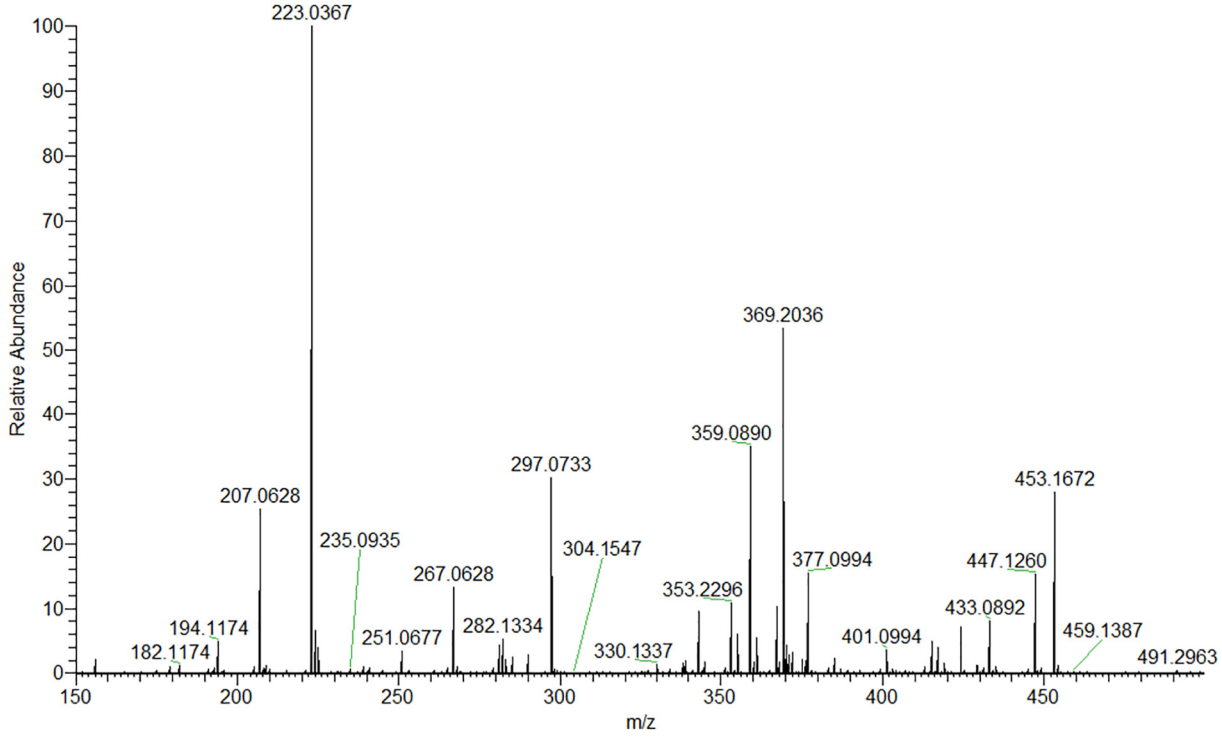

Fig. 3. ESI-HR mass spectrum of the polar fraction containing ions at *m/z* 207.0628 and 223.0367.

compound is also known under the name of tyrosol, which is one of the main phenolic constituents of olive oil (Romero and Brenes, 2012). Mass spectrum of the next abundant peak (RT 9.14 min) contains a possible molecular ion of *m/z* 184 (Fig. 2C). An argument towards this prediction is given by the ESI-HRMS spectrum, which contains ions at *m/z* 207.0628 and 223.0367 with elemental composition $C_9H_{12}O_4Na$ and $C_9H_{12}O_4K$, respectively (Fig. 3). Thus, the molecular formula of the compound is $C_9H_{12}O_4$. Such molecule would have a molecular ion at exactly *m/z* 184 in EI mass spectrum, resulting in a proper correlation between the two ionization methods. The observed fragmentation pattern corresponds to the structure of a phenolic compound related to tyrosol. The absence of $[M-31]^+$ ion peak signifies a different arrangement of the alkyl substitute comparing to tyrosol. The most probable structure is that of 2-methoxy-4-ethoxyresorcinol or 3-methoxy-4-ethoxycatechol.

The minor peaks in the chromatogram (Fig. 2A) corresponding to a group of phenolic compounds were identified as 2-methoxy-4-(2-hydroxyethyl)phenol (RT 7.98 min), 4-(2-hydroxyethyl)catechol (RT 8.67 min) and hydroxyl-methoxybenzyl alcohol (RT 7.46 min). Two other groups were detected with GC–MS at RT 7.11 min and RT 7.21 min and assigned to the class of terpenes and terpenoids. Unfortunately, as these compounds are less amenable to ESI, there were no signals correlating to the spectra observed in EI mode. Low-resolution GC–MS did not allow us to identify their structures. In addition to data obtained with GC–MS, several compounds with high unsaturation degree were detected by ESI-HRMS: *m/z* 267.0628 ($C_{14}H_{12}O_4Na$ – 0.15 ppm), *m/z* 297.0733 ($C_{15}H_{14}O_5Na$ – 0.05 ppm), *m/z* 359.0890 ($C_{20}H_{16}O_5Na$ – 0.07 ppm), *m/z* 369.2036 ($C_{21}H_{30}O_4Na$ – 0.19 ppm), 377.0994 ($C_{20}H_{18}O_6Na$ – 0.30 ppm), *m/z* 433.0892 ($C_{22}H_{18}O_8Na$ – 0.55 ppm), *m/*

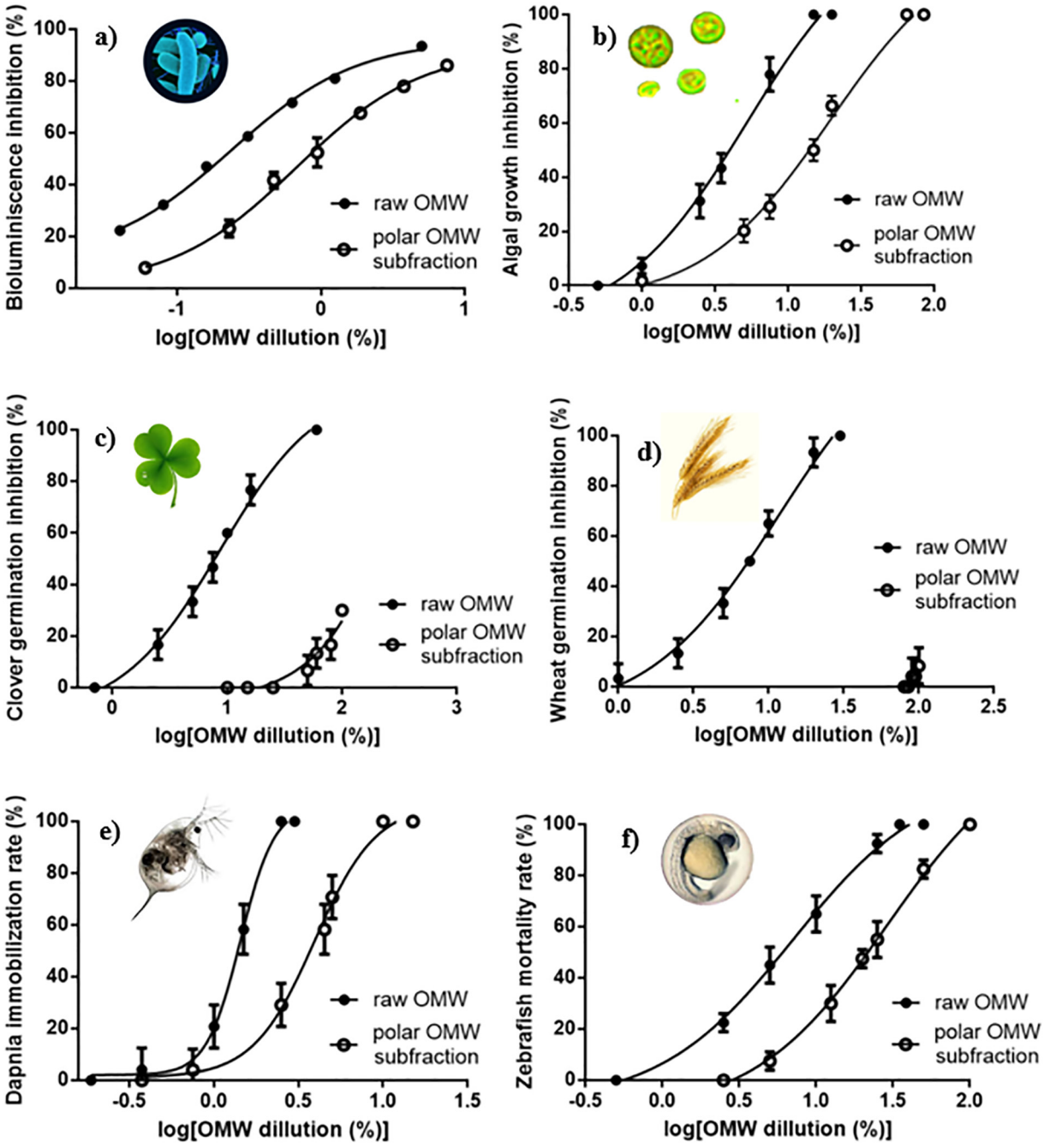

Fig. 4. Concentration-response curves representing the toxic effect of raw OMW and polar fraction on: a) bioluminescence of bacteria *V. fischeri*, b) *C. vulgaris* growth, germination of clover *T. repens* (c) and wheat *T. aestivum* (d), *D. magna* mobility (e) and *D. rerio* survival (f). Percentage (%) of values (mean ± SD) is shown versus the logarithm of the OMW dilution.

z 447.1260 ($C_{20}H_{24}O_{10}Na$ – 0.40 ppm), *m/z* 453.1672 ($C_{27}H_{26}O_5Na$ – 16 ppm) (Fig. 3). Considering the unsaturation degree from 8 up to 15 and the number of oxygen atoms, phenolic structures or phenols with unsaturated alkyl chain could be proposed.

3.3. Ecotoxicity tests

3.3.1. Acute toxicity of raw OMW and polar fraction

Results of toxicity tests are presented as concentration-response curves which were used for the calculation of EC_{50} values for each model organism (Fig. 4, Table 3). Tested samples demonstrated a time- and dose-dependent mode of action. Taking into account obtained values, raw OMW was 2.75–4.11 times more toxic to all model organisms comparing to the polar fraction (Table 3). The highest toxic effect was observed on *V. fischeri*, as only 0.24% of raw OMW and 0.67% of polar fraction induced 50% of bioluminescence inhibition (Table 3). Only the polar fraction in germination tests did not induce any significant toxic effects. After 120 h of exposure, EC_{50} was not reached since undiluted sample of polar fraction only inhibited the germination of 12.5% clover seeds and 28.0% of wheat seeds.

3.3.2. Sub-acute toxicity of raw OMW and polar fraction

In order to achieve a better understanding of OMW toxicity potential, additional sub-lethal endpoints were observed during germination seed test and ZET.

3.3.2.1. Growth inhibition test. Seeds plant and roots length were measured to observe OMW impact on vegetative growth. Polar fraction had a low impact on seeds germination, while a high negative impact on plant and root elongation was observed (Fig. 5; Table 4). Wheat and clover root, as well as wheat sprout length, decreased with higher concentrations of raw OMW or polar fraction. This decrease of roots length was more evident with clover (only 3.52% of raw OMW induced toxic effect at 50% of the sprouts; Table 4). At the same time, only 0.38% of the raw OMW and 11.74% of the polar fraction caused 50% inhibition of wheat sprout elongation. Additionally, roots waiving and coils were observed after exposure to OMW and polar fraction, but their formation was not concentration dependent.

3.3.2.2. Zebrafish embryotoxicity test. Both, raw OMW and polar fraction induced numerous effects during zebrafish ontogenesis. Based on the high lethality of *D. rerio*, additional effects were observed during exposure to the sub-lethal OMW– abnormality and hatching rate, as well as pigmentation formation. Tested samples induced negative impact during zebrafish ontogenesis, even at low concentrations (Fig. 6; Table 4). Raw OMW at concentration of 11.98% induced 50% of developmental abnormalities (Table 4). In the first 96 h of exposure strong developmental retardations [developmental delay (Fig. 6e and f), absence/delay in pigmentation formation (Fig. 6: g–l)] were noted. Interestingly, although polar fraction induced 5.16 times lower impact on embryo development (Table 4), almost the same abnormalities were observed on both tested samples. Thus, both samples induced pigmentation formation (Fig. 6: g–l) and caused pericardial (Fig. 6: i–l) and yolk sac (Fig. 6l) edema, as well as blood accumulation at the yolk sac (Fig. 6: i, k, l). Among observed endpoints, hatching appeared as the most sensitive (only 3.28% of the raw OMW induced hatching of 50% specimens; Table 4). At 96 hpf, hatching occurred in 100% of the survived control specimens. Nervous behavior was observed among unhatched fish, which manifested through twitching of the body.

4. Discussion

The composition of OMW is very variable and depends on multiple factors such as extraction process, fruit ripeness, olive variety, but also on physical and chemical characteristics of OMW (Justino et al., 2012; Pardo et al., 2017). The sample tested within this study had similar characteristics to other OMW samples through the literature that were also characterized as a dark-colored sample caused by lignin polymerization with phenolic compounds, with a typical odor and increased acidity (El-Abbassi et al., 2017; Justino et al., 2012). Inorganic content of OMW was mainly composed of metals, which are related at the same time to nutritional benefits (i.e. iron, copper, zinc) and toxicological impact. Concentrations of the most abundant metals reported in this study were about 10 times higher for iron and about 2.5 times higher for zinc, comparing to those already reported in the literature (Chatzistathis and Koutsos, 2017; El-Abbassi et al., 2017). Such a high concentration of iron and zinc could be explained through different manufacturing processes that are applied. At the same time, concentrations of other metals were similar or lower than those reported (Chatzistathis and Koutsos, 2017; El-Abbassi et al., 2017).

The Folin-Ciocalteu procedure (Vitali Čepo et al., 2017) revealed our samples as highly enriched in phenolic compounds (9.22 ± 0.17 g GAEa L^{-1}) when comparing to the previous studies, which mainly reported phenolic concentrations up to 10.7 g L^{-1} (Pardo et al., 2017). According to the Urban Water Directive (Directive, 1991), the maximum amount of phenols in wastewater is set to <1 mg/L, which is one of the main reasons why OMW tested within this study should not be released directly into the sewer systems or water bodies. Moreover, due to the increased BOD_5/COD ratio (Table 2) mainly caused by the high amount of phenolic compounds, OMW tested within this sample can be characterized as medium biodegradable ($0.2 < BOD_5/COD < 0.33$; Bouknana et al., 2014). Such pollution properties should be taken into account during selection of appropriate OMW treatment method and before OMW releasing into the receiving systems.

The identification of individual phenolic compounds by GC–MS pointed out tyrosol and catechol as the most abundant phenolic compounds. Both phenolic compounds are commonly found in OMWs and are well known for their antioxidant activities (Gómez-Caravaca et al., 2014), as well as for their toxic potential (Justino et al., 2012). Previous studies recorded synergism between some phenolic compounds (hydroxytyrosol and gallic acid), but also among phenols and other bioactive compounds (hydroxytyrosol and ascorbic acid), therefore emphasizing their unpredictable effect on environmental organisms (Justino et al., 2012; Tafesh et al., 2011).

Although the chemical characterization of OMW gave us an insight into the mixture of compounds present in this complex matrix, accurate

Table 3
Overview of the EC_{50} acute toxicity values obtained during exposure of the model organisms to the raw OMW and their polar fraction.

Test performed	Species used	Exposure period (h)	Raw OMW		OMW polar fraction	
			EC_{50} (% of dilution)	95% confidence intervals	EC_{50} (% of dilution)	95% confidence intervals
Bioluminescence test (ISO 11348-3, 2007)	*V. fischeri*	0.5	0.24	0.21–0.28	0.67	0.506–0.889
Algal growth inhibition test (OECD 201, 2011)	*C. vulgaris*	72	5.20	3.56–7.60	19.30	14.45–25.78
Seed germination test	*T. repens*	120	8.68	6.76–11.15	*	*
	T. aestivum		11.58	8.02–16.73	*	*
Acute immobilization test (OECD 202, 2004)	*D. magna*	48	1.43	1.33–1.55	3.93	3.51–4.41
ZET test (OECD 236, 2013)	*D. rerio*	92	7.05	5.26–9.45	28.96	21.21–39.55

* 50% of mortality not observed even on undiluted samples.

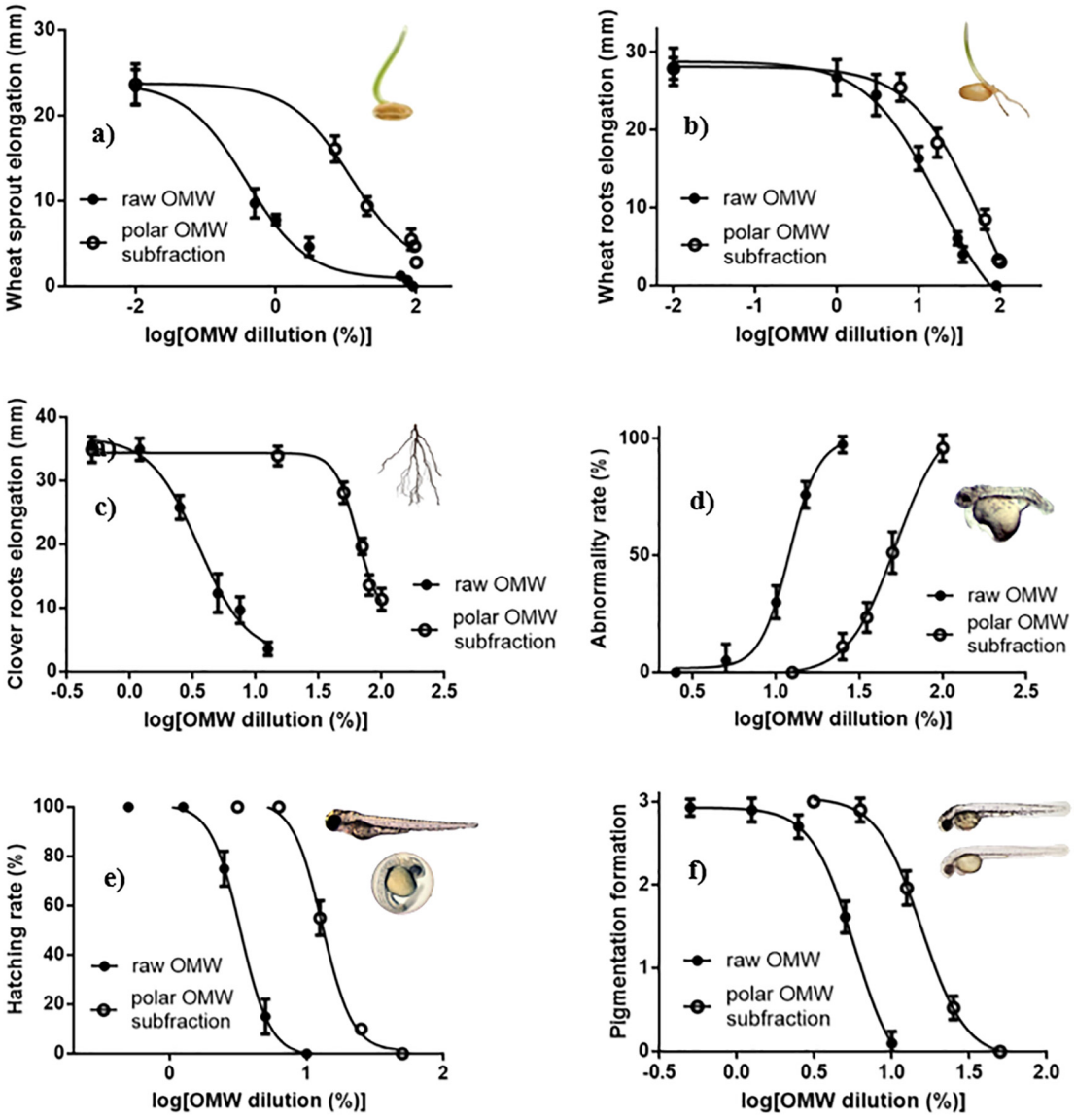

Fig. 5. Concentration-response curves representing the sub-acute toxicity of raw OMW and polar fraction on: wheat *T. aestivum* (a) sprout and (b) roots elongation; clover *T. repens* (c) roots elongation; zebrafish *D. rerio* a) development, b) hatching success and c) pigmentation formation. Percentage (%) of values (mean ± SD) is shown versus the logarithm of the OMW dilution.

assessment of OMW toxicity can only be achieved by determining the impact on different model organisms. Previous studies that evaluated toxic effects of OMW were mainly based on determining the impact on seed germination and plant growth (Chatzistathis and Koutsos, 2017; Massoudinejad et al., 2014; Rusan et al., 2015) and less often on the overall impact at the whole organism level using different bioassays (Hrubik et al., 2016; Justino et al., 2012). Moreover, most of the studies did not observe sub-lethal endpoints that within this study showed to be crucial for understanding OMW-induced toxic effects on plants, invertebrates and vertebrates. In our study, bioluminescence test on bacteria *V. fischeri* resulted in EC_{50} values for raw OMW (0.24%) and polar fraction (0.67%) which agree well with previous studies on OMW toxicity (Amaral et al., 2012; Mekki et al., 2008). Strong sensitivity of *V. fischeri* and high toxicity of polar fraction suggest that the effects of OMW are mainly attributed to phenolic load. Our findings are also in line with the study reporting bioluminescence inhibition mainly as a

Table 4
Overview of the EC_{50} sub-acute toxicity values obtained during exposure of the clover *T. repens*, wheat *T. aestivum*, and zebrafish *D. rerio* to the raw OMW and their polar fraction.

Test performed	Observed endpoint	Exposure period (h)	Raw OMW		OMW polar fraction	
			EC_{50} (% of dilution)	95% confidence intervals	EC_{50} (% of dilution)	95% confidence intervals
Seed germination test	*T. repens* root elongation	120	3.52	2.91–4.27	64.23	57.03–72.34
	T. aestivum sprouts elongation		0.38	0.29–0.50	11.74	7.20–19.12
	T. aestivum root elongation		17.29	11.92–25.08	56.61	45.23–80.96
ZET test (OECD 236, 2013)	*Developmental abnormalities*	96	11.98	10.85–13.23	61.87	39.95–77.95
	Hatching		3.28	2.93–3.67	13.08	12.09–14.16
	Pigmentation		5.72	4.26–7.68	15.50	13.68–17.57

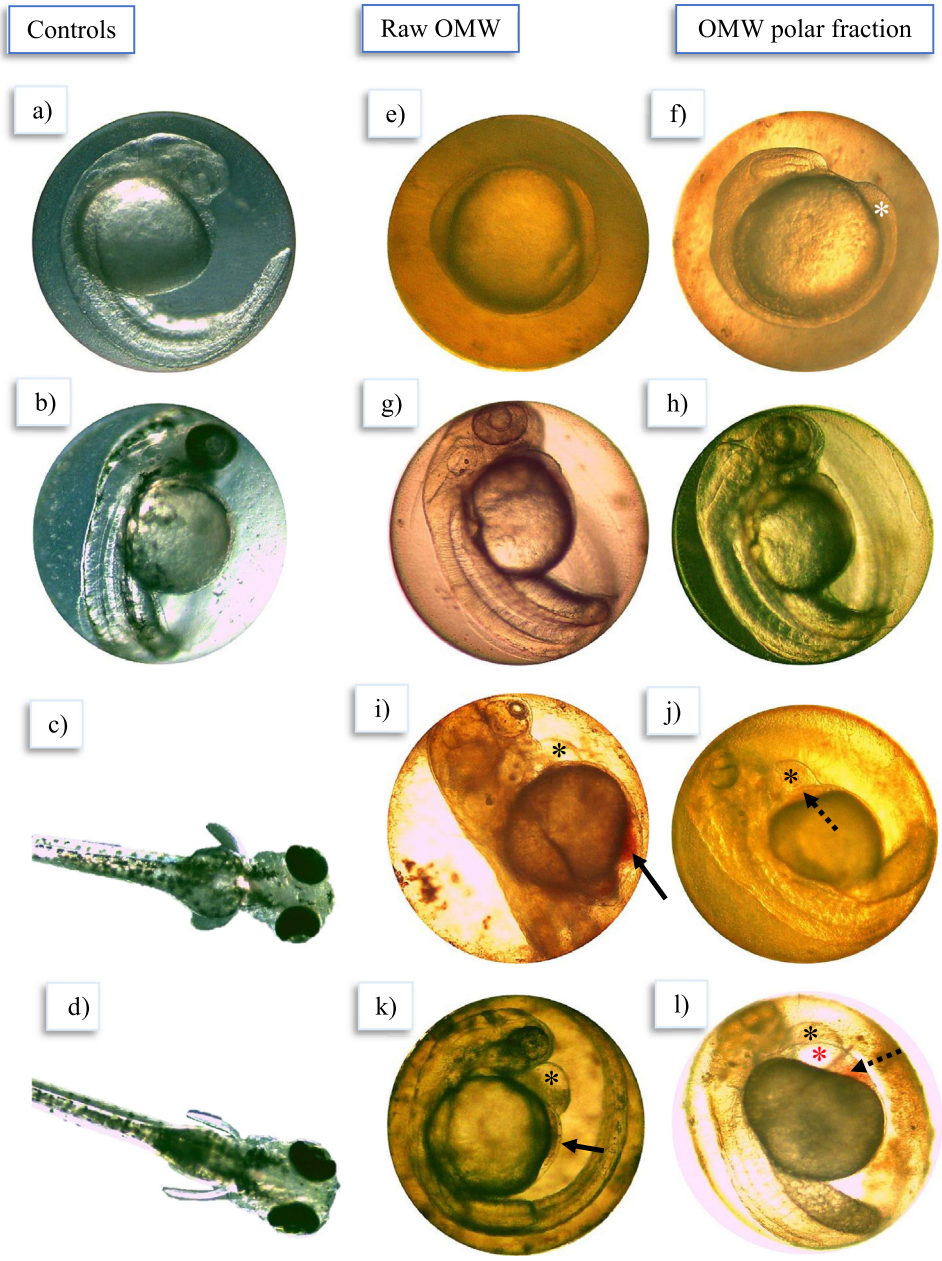

Fig. 6. Representative pictures of zebrafish embryos exposed to a dilution series of raw OMW and their polar fraction. Pictures marked as a), b), c), d) represent controls at 24, 48, 72 and 96 hpf, respectively. Tested samples caused: e) and f) developmental retardation, non-detachment of the tail (white asterisk); g) and h) lack in pigmentation formation; i) and j) pericardial edema (asterisk), major (arrow) and minor (dashed arrow) blood accumulation at the yolk sac; k) and l) pericardial edema (black asterisk), minor (arrow) and major (dashed arrow) blood accumulation at the yolk sac, yolk sac edema (red asterisk), lack in pigmentation formation. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

result of tyrosol, hydroxytyrosol and catechol presence in tested samples (Mekki et al., 2008).

Regarding the microalgae, it is important to underline that they are mainly incorporated in bioremediation processes and less in evaluation of OMW toxicity (Andreozzi et al., 2008; DellaGreca et al., 2001; Fiorentino et al., 2003). *C. vulgaris* used within this study showed great potential regarding high sensitivity to a variety of toxicants present in raw OMW and polar fraction (EC_{50} = 5.20% and 19.30%, respectively). Obtained results were in accordance with the previous studies on algae reporting high toxicity of low molecular weight (350 Da) phenols, such as catechol and hydroxytyrosol, on freshwater microalgae *Pseudokirchneriella subcapitata* (Andreozzi et al., 2008; Fiorentino et al., 2003) and *Ankistrodesmus braunii* (DellaGreca et al., 2001). Regarding the fact that raw OMW strongly inhibited *C. vulgaris* growth, the impact of heavy metals should not be excluded. Ouyang et al. (2012) already recorded that copper, chromium, zinc, cadmium, lead have the potential to inhibit the growth of *C. vulgaris*, even at low concentration (0.5–5 μM).

In addition, we have showed that OMW, in particular the polar fraction, caused significant negative impacts not only on microorganisms (bacteria, algae), but also on microcrustaceans *D. magna* (EC_{50} = 1.43% and 3.93%, respectively) and vertebrate zebrafish *D. rerio* (EC_{50} = 7.05% and 28.96%, respectively). Fiorentino et al. (2003) reported catechol and hydroxytyrosol as the most toxic phenolic compounds to *D. magna*. Regarding zebrafish, only one study (Rouvalis et al., 2013) implemented this model organism into their experiments with OMW and pointed out the high toxicity potential of tested raw sample (EC_{50} = 0.48%). However, Rouvalis et al. (2013) did not assess the sub-lethal

effects which resulted in particularly interesting and useful observations in this study, therefore proving to be a very valuable addition to standardized embryotoxicity test. Abnormalities observed during exposure to raw OMW and polar fraction were very similar (non-hatching, pericardial edema, blood accumulation), but more pronounced during exposure to raw sample (EC_{50} = 11.98%), when compared to polar fraction (EC_{50} = 61.87%). Wu et al. (2014) showed that heavy metals present in urban highway runoff can cause pericardial edema, blood clotting, non-separating of the tail from the yolk sac and yolk sac edema, that were also the most pronounced abnormalities observed in zebrafish exposed to the raw OMW. Similar abnormalities were also noticed during embryo exposure to manganese and lead, but in much higher concentrations (25–100 μM and 2.5–10 μM, respectively) than detected in our OMW sample (Tu et al., 2017). Moreover, hatching and pigmentation formation was also altered. Those findings can be related to several studies (Jezierska et al., 2009; Tu et al., 2017; Wu et al., 2014) that pointed out the potential of zinc, copper, manganese and cadmium to delay and/or lower hatchability, while cadmium and copper are well known about their potential to inhibit tyrosinase activity and prevent/delay formation of melanin (Carlsson et al., 2014). Considering their ability to pass through the zebrafish chorion and promote developmental abnormalities during organogenesis (Jezierska et al., 2009; Wu et al., 2014), heavy metals might be the key pollutants in raw OMW that influenced zebrafish development. Though metals were not solely responsible for all observed endpoints, their interaction with present phenolic compounds in OMW complex samples could result in combined effects and OMW increased toxicity potential.

To date, phytotoxic effect of raw OMWs on a wide range of cultivated plants, such as barley, chicory, tomato, wheat was reported from different authors (Casa et al., 2003; Komilis et al., 2005; Rusan et al., 2015). Those studies assumed that phenolic compounds are the main cause of phytotoxicity (Isidori et al., 2005), while data presented within our study showed that OMW's phytotoxicity is much more complex than previously considered. Both clover and wheat showed high sensitivity to raw OMW sample (EC50 = 8.68% for clover and 11.58% for wheat), suggesting that OMW land disposal would strongly affect newly grown seeds. At the same time, the corresponding concentration of undiluted polar fraction did not inhibit germination in such a high percentage. We observed approximately 30% of clover and 10% of wheat germination inhibition. According to this data, phytotoxic properties are probably related to the osmotic and ion-toxicity effects of the salts contained in the raw OMW (Rusan et al., 2015). Sodium chloride is added during the industrial processes for preservation purposes. It is dependent on the method of conservation of olive fruit before milling, but usually, those values varied from 0.4 to 2.5% (El-Abbassi et al., 2017). Raw OMW had high toxicity to the vegetative growth which can also be explained through salt stress that resulted in reduced cell turgidity and inhibition of root and sprout elongation. Ramana et al. (2002) also reported a decrease in germination rate of tomato, cucumber, onion, chilly and bottle gourd, as a result of an increase in concentration of the highly saline effluent (EC = 25.3 dS m^{-1}). Sub-acute effects of polar fraction showed inhibition/decrease of sprouts (clover) and roots elongation (clover and wheat), potentially related to the toxic action of present phenolic compounds.

Applied bioassays allowed us to test the species-specific toxicity of OMW and to slightly elucidate common mechanisms of OMW mode of action at the whole organism level. The obtained results indicated *V. fischeri* as the most sensitive model organism and potential model organism for OMW early-pollution monitoring. Interestingly, plants, although most frequently used in determination of OMW toxicity, showed to be the least sensitive. Such findings emphasize the need for testing the potential impacts at different trophic levels and different complexity. This study highlighted the polar fraction as the main carrier of adverse effects observed on most model organisms, which leads to a conclusion that OMW treatments that are based only on phenolic compounds removal are not sufficient to reduce their toxic potential. Besides aromatic compounds, OMWs contain other organic molecules, including nitrogen compounds, sugars, organic acids, and pectins (Bouknana et al., 2014), that increase OMW's organic load. In addition, high concentrations of potassium, magnesium, and phosphate salts, as well as pH (Bouknana et al., 2014), should be monitored during treatment of OMW samples before disposal. For that reason, one of the highest priorities in this field should be sustainable environmental management, which could be achieved through improving already existing treatment technologies, while giving special attention towards compounds present in the polar fraction and their chemical characterization.

5. Conclusion

Data obtained within this study showed that raw OMW and its polar fraction are highly toxic not only to microorganisms but also to plants, invertebrates, and vertebrates. Since toxicity retained even at low OMW concentrations, dilution of OMW should not be recommended as a possible solution prior to the disposal of OMW. For that reason, one of the most challenging future priorities should be the establishment of guidelines to manage OMW through appropriate treatment methods (with the special emphasis to the polar fraction and its components) in order to minimize the environmental impact. One of the main goals should be to set up standardized bioassays that will verify both the treatment method efficacy, but also the toxic potential of OMW effluents. Such evaluation could only be achieved through the usage of multi-model organisms from different trophic levels with different sensitivities. Bioassays conducted within this study could enable fast, simple and reliable monitoring of one of the most troublesome agro-industrial effluents (OMW), and should be considered in order to maintain the sustainability of receiving ecosystems.

Declaration of Competing Interest

There are no conflicts of interest.

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