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Effects of Olive Oil Mill Waste Water (OMWW) on the Frog Larvae

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Abstract In this research, acute effect of the olive oil mill wastewater (OMWW) on the frog larvae has been studied. Larvae showed hyperactivity symptoms first and loss of balance and remained motionless due to toxicity of wastewater. Toxicity was observed between 2 and 159 min depending on the test concentrations. Upon removing the phenolic compounds from the OMWW, this effect was seen after 248 min. Potential effects of the OMWW in Lake Iznik were also researched. Salinity of the lake water changed from 0.2 ‰ to 0.0 ‰ respectively in the measurements done in May and December.

Keywords OMWW · Olive oil mill wastewater · Frog larvae

Amphibian populations are declining globally at an alarming rate (Wake 1991; Houlahan et al. 2000). The mechanism responsible for these declines includes various chemical pollution caused by pesticides, herbicides, fertilizers, destruction of habitats and global climate change (Sparling et al. 2001; Blaustein and Kiesecker 2002; Khan and Law 2005; Koponen et al. 2007). The skin of a frog is very permeable (Bentley and Yorio 1977) and the tadpole stage also relies on gills for breathing (Gargaglioni and Milsom 2007), both of these physiological characteristics make frogs and tadpoles very susceptible to a number of contaminants. Consequently, because frogs inhabit both in water and land, they are more likely to be exposed to environmental contaminants compared to many other animals.

Lake Iznik is one of the major habitats of the frogs. It is the fifth biggest in Turkey, surrounded by agricultural lands, orchards, several villages and two towns, one of which is Iznik (Niceae) (Papadimitriou et al. 1997; Ozeren 2009). *Olea europaea* L. is a typical agricultural crop of and is widely cultivated in the Lake Iznik region for oil production and consumption as a natural fruit. Due to this, there are several olive oil production plants around Lake Iznik for the extraction of oil from the olives produced in that region. The majority of these olive mills are small scale businesses, which cannot afford the high capital cost required for proper wastewater treatment. As a result, they often resort to dumping their wastewater, untreated, around the region. In our study, we have characterized the water quality of Lake Iznik and the acute toxicity of OMWW to frog larvae using controlled laboratory experiments. Additionally, we have examined Secchi disk measurement and pH, redox potential, dissolved oxygen (DO) content, salinity and conductivity of Lake Iznik to determine its current water quality.

Materials and Methods

The current study was approved by Istanbul University Local Committee on Animal Research Ethics. Totally, 42 frog larvae (*Pelophylax ridibundus*) at the stage of 28 and for each test, six larvae were used in this study. They were kept in plastic containers in aired tap water and fed daily with boiled lettuce in the water which was replaced every second day. The stage of the larvae used in pre-metamorphic

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stage was determined according to Gosner's staging table (Gosner 1960). Six frog larvae were used for each test and mean average of impairment in motility was calculated to be able to compare the effect of pollution on the tested larvae. Of these, eighteen larvae were used in order to observe the behavioral change of the tested larvae in 400 mL of 10 %, 25 % and 50 % OMWW containing polluted waters. Signs of toxicity were determined first by hyperactivity symptoms, then loss of balance and motionlessness. After the motionless state of the toxicity symptoms, all the larvae were removed immediately from the solution and placed into the clean water having high oxygen content without killing the tested animals. In the following experiment, six larvae were tested in 400 mL of OMWW containing polluted water after the removal of phenolic compounds with the extraction method using ethyl acetate (EtAc) as the solvent. Finally, eighteen larvae were used to test the DO, pH and salinity parameters one by one on the frog larvae, since these are the important OMWW containing water quality parameters.

Extraction of OMWW was done by using EtAc, supplied by Merck Company and was used directly as it was without further treatment. An equal volume of OMWW and distilled water were put in a beaker and was left to mix for an hour on a magnetic stirrer plate. Organic layer was removed from the OMWW, and this extraction was repeated in order to remove all the EtAc soluble compounds from the OMWW. Then, EtAc solvent was removed from the solution under vacuum by heating gently at 40°C. Obtained EtAc soluble compounds were used in HPLC analysis for the detection of oleuropein and tyrosol antioxidant compounds. After the removal of EtAc soluble compounds, remaining OMWW were kept under vacuum for 3 h to remove the dissolved EtAc within the waste solution. Then, this waste solution was used in the toxicity test to be able to observe the effect of OMWW without phenolic ingredients on the tested larvae.

Lower DO ratios on the larvae were tested by using nitrogen (N_2) gas, bubbled through the solution to decrease the dissolved oxygen to the required level. Measurement of the dissolved oxygen (DO) level was also performed at the same time to adjust the DO amount. Salinity of the test water was adjusted by using sodium chloride (NaCl) supplied from Carlo Erba and WTW 340i multimeter. pH of the test solution was adjusted by using hydrogen chloride (HCl) supplied by Aldrich and WTW 340i multimeter.

Temperature, pH, oxidation–reduction potential (ORP), DO, and electrical conductivity were measured by using a WTW 340i multi-meter. Water quality parameters of OMWW of different concentrations in Lake Iznik were measured after the calibration of each parameter. The calibration of each parameter was checked before the measurements, and multi-meter was calibrated to 2 pH standards (4.00 and 7.00), and a specific conductance standard was used (1413 µS/cm) prior to use each day. The dissolved oxygen meter was calibrated to 100 % oxygen (atmospheric conditions). Initial and ongoing calibration checks were made throughout each sampling day.

Secchi depth was measured with a 20 cm diameter disk colored both white and black at the Iznik town station. Secchi depth was calculated as the mean of the depth of disappearance and reappearance. The disk was viewed from the shady side of the boat to reduce surface reflectance, but there was always sufficient clarity that the disk was fully illuminated by sunlight passing beneath the boat.

A reversed-phase high-performance liquid chromatographic (HPLC) technique was used to identify and quantify the tyrosol and oleuropein compounds found in the OMWW extract. For this purpose, standard solutions of these phenolic compounds were used for the comparison. Sample concentrations were calculated, based on peak areas compared to each of these external standards. A Schimadzu chromatograph was used, and it was equipped with ODS hypersil (4.6–250 mm) column, LC-10AT pump and SPD-10 Avp detector. The flow rate was 1 mL/min. and temperature was maintained at 30°C during the analysis. 10 µL of extract was injected, and the mobile phase used was 50 % water and 50 % methanol (MeOH) for 10 min. in isocratic mode. After that, only MeOH was used as the mobile phase for 5 min. for a total running time of 15 min.

Results and Discussion

In order to observe the effects of tested water quality parameters on the frog *Pelophylax ridibundus* larvae, OMWW in different concentrations was prepared and measurement results of all these parameters were recorded as in Table 1.

Obtained results clearly indicate that OMWW is reducing the DO level and pH of the contaminated water whereas it is causing an increase in the ORP, conductivity and salinity values. We also tested these parameters to be able to see the effect of EtAc extraction or removal of the phenolic compounds on the OMWW water quality. Thus, we would be able to see the contribution of the phenolic ingredients on the tested water quality parameters as in Table 2.

After the removal of phenolic ingredients from the OMWW, oxygen level and pH values increased from 0.7 % to 1.9 % and from 4.84 to 5.06 respectively. ORP value of the OMWW (97 mV) decreased even more drastically after the extraction of OMWW with EtAc solvent (85 mV). OMWW having the concentration of 25 % (v/v) had the same ORP value. Obtained results clearly indicate

Table 1 Water quality parameters of OMWW in different concentrations

OMWW (%) (v/v)	°C	O_2 (mg/L)	O_2 (%)	pH	ORP (mV)	Cond.	Sal. (%)
0	24.9	3.05	37.0	6.92	−25	482 µs/cm	0.0
10	24.6	1.75	21.0	5.26	69	20.4 ms/cm	12.2
12.5	24.8	1.66	20.2	5.19	76	27.3 ms/cm	16.8
25	24.9	0.83	10.0	5.06	85	48.7 ms/cm	31.8
50	25.1	0.14	1.7	5.02	88	85.6 ms/cm	60.9
75	25	0.06	0.8	4.95	91	113.4 ms/cm	OFL
100	25.2	0.05	0.7	4.84	97	138.8 ms/cm	OFL

ORP oxidation reduction potential, *Cond.* conductivity, *Sal.* salinity, *OFL* off line

Table 2 Water quality parameters of OMWW after extraction with EtAc

OMWW (%) (v/v)	°C	O_2 (mg/L)	O_2 (%)	pH	ORP (mV)	Cond. (ms/cm)	Sal. (%)
100	25.3	0.15	1.9	5.06	85	127.8	OFL

ORP oxidation reduction potential, *Cond.* conductivity, *Sal.* salinity, *OFL* off line

that antioxidant compounds affect the water quality parameters quite negatively.

There are a lot of literature studies on the presence of antioxidant compounds found in OMWW (Mulinacci et al. 2001; Ranallı et al. 2003). According to the past studies on the examined waste, *tyrosol* and *oleuropein* are two of the common antioxidants in OMWW (Visioli et al. 1995; Roig et al. 2006). Thus, after extracting the OMWW with EtAc, we tested the presence of the chemicals mentioned in the extract by using an HPLC instrument (Figs. 1 and 2).

Above chromatograms proved that the characteristic properties of the OMWW used were the same as in the other studies mentioned. Additionally, this test proved that phenolic antioxidant molecules present in OMWW were removed after the extraction process as expected.

In the second part of this research, toxicity tests were carried out in our laboratory and different concentrations of OMWW were tested in order to see the effect of pollution on the behavioral change of larvae. To our knowledge, this is the first detailed toxicology study done on the frog larvae with OMWW.

Tested larvae were kept in 400 mL of polluted waters containing 10 %, 25 % and 50 % OMWW. It was found that OMWW was causing impairment in the motility of the larvae and the toxicity symptoms as mentioned in the literature (Sayım and Kaya 2006) after a certain period of time depending on the concentration.

In our first toxicity test, all the larvae were affected by the 50 % OMWW containing water approximately within 2 min (Table 3). Then, we tested the effect of 25 % OMWW and impairment in motility was observed within 6.08 min. Finally, 10 % OMWW was tested and decrease of concentration from 25 % to 10 % caused an increase in

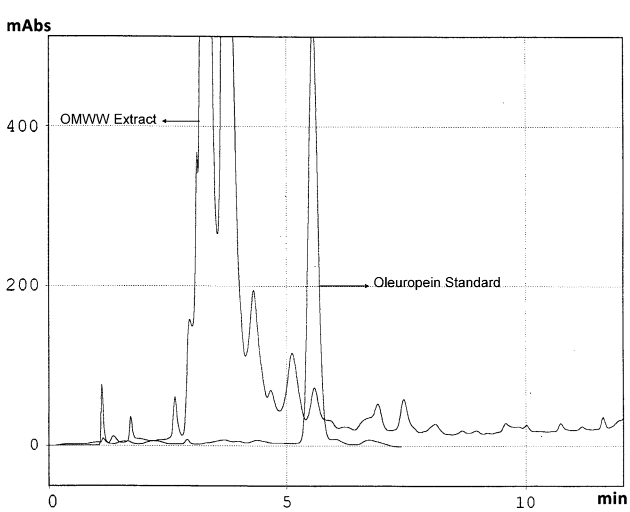

Fig. 1 HPLC chromatogram showing the presence of *Oleuropein* in OMWW

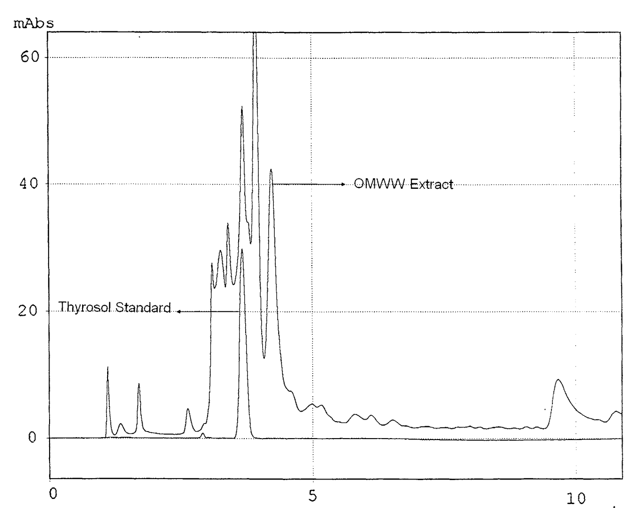

Fig. 2 HPLC chromatogram showing the presence of *Tyrosol* in OMWW

the toxicity duration. As a result of this concentration change, toxicity effect was observed after 2 h and 39 min. All OMWW exposed larvae were characterized by hyperactivity symptoms at the beginning of the experiment. Then, loss of balance and remaining motionlessness were seen in all these larvae.

In this research, we also tested the toxicity effect of phenolic compounds found in the OMWW. Upon removing these antioxidants, impairment in motility was observed on the larvae individuals after 4 h and 8 min. To our knowledge, this is the first comparative toxicity experiment conducted by using OMWW with and without extracting the phenolic ingredients in it.

In our final test, we determined the toxicity effect of each susceptible parameter on the larvae. DO, pH and salinity were tested one by one considering each parameter as the potential candidate causing toxicity. Salinity was tested in 12.2 ‰ saline water and impairment in motility on the tested larvae could only be seen after 9 h and 10 min. Obtained result clearly showed us that the water salinity was adversely affecting the frog larvae. Second group were kept in a medium having a pH of 4.84. After 3 days of observation, pH didn't cause any symptom of toxicity. Although DO was observed as the most stress causing parameter on the larvae during the final study, we couldn't manage to adjust the dissolved oxygen level to about 20 %. During this test, all the larvae remained motionless at the beginning of this experiment.

In the last part of this study, potential effects of the OMWW in Lake Iznik were researched. Measured parameters in the field included temperature, pH, and specific conductance and dissolved oxygen (Tables 4 and 5). Measurement of these parameters was done twice in a year in order to compare the obtained values at different seasons. Temperatures ranged from 19.7 to 16.3°C and higher temperature values were obtained in May than the ones in December. The pH values were all basic and ranged from 8.66 to 9.03. A big change in specific conductance and salinity values were observed from May to December. Specific conductance value decreased from 963 µS (microsiemens)/cm to 113 µS/cm and salinity value changed from 0.2 % to 0.0 %. Dissolved oxygen varied

Table 3 Laboratory exposure conditions of the tested larvae

Test medium	Number of tested larvae	Mean average of toxicity duration	Number of respondents	Maximum test duration
50% OMWW	6	2 min.	6	2.2
25% OMWW	6	6.08 min.	6	6.10 min.
10% OMWW	6	2 h. and 39 min.	6	2 h. and 42 min.
100% OMWW after the removal of phenolics	6	4 h. and 8 min.	6	4 h. and 12 min.
12.2‰ Saline water	6	9 h. and 10 min.	6	9 h. and 18 min.
pH 4.84	6	3 days	0	3 days

Table 4 Measurement results of field parameters performed in Lake Iznik on May 6, 2010

Location	°C	O_2 (mg/L)	O_2 (%)	pH	ORP (mV)	Cond.	Sal. (‰)
Boyalıca V. 40°23′08,12″N 29°31′57,94″E	17.7	16.21	174.1	9.03	−147	958 µs/cm	0.2
Muskule V. 40°23′08,12″N 29°31′01,31″E	19.3	13.32	143.2	8.71	−132	963 µs/cm	0.2
Iznik Town 40°26′13,19″N 29°42′56,27″E	19.7	13.86	148.1	8.66	−122	962 µs/cm	0.2

ORP oxidation reduction potential, *Cond.* conductivity, *Sal.* salinity

Table 5 Measurement results of field parameters performed in Lake Iznik on December 1, 2010

Location	°C	O_2 (mg/L)	O_2 (%)	pH	ORP (mV)	Cond.	Sal. (‰)
Boyalıca V. 40°23′08,12″N 29°31′57,94″E	16.3	10.22	105.6	8.96	−98	113 µs/cm	0.0
Muskule V. 40°23′08,12″N 29°31′01,31″E	16.8	9.01	94.3	8.85	−88	115 µs/cm	0.0
Iznik Town 40°26′13,19″N 29°42′56,27″E	17.1	9.84	98.9	8.83	−84	116 µs/cm	0.0

ORP oxidation reduction potential, *Cond.* conductivity, *Sal.* salinity from 10.22 to 16.21 mg/L and oxygen levels were lower in December than in May (Tables 4 and 5).

Briefly, decrease in the pH and DO and increase in the salinity, conductivity and oxidation reduction potential—values were observed in the waters contaminated with OMWW. In addition to that, OMWW containing waters caused impairment in motility in the frog larvae after a certain time period. Increase in OMWW pollution in water decreased the duration of observation of the impairment in motility of the larvae. Toxicity of pH and salinity parameters were also tested on the frog larvae to better understand the main cause of the impairment in motility for this species. It was clear that polluted OMWW containing water had a negative effect on the larvae in a much quicker time period than each parameters of OMWW (salinity, DO content and pH) alone. The water quality parameters in Lake Iznik were also tested both in May 2010 and December 2010. Salinity and conductivity parameters of the lake were quite different when obtained results were compared to each other. These measurement differences could be due to the OMWW pollution on the lake water.

Organic acids found in the OMWW are lowering pH value and antioxidant phenolic ingredients together with the other pollutants having an adverse effect on the DO value of the polluted water. Although all the tested parameters in the field may easily get affected from other external sources, monitoring chemical oxygen demand (COD) and biological oxygen demand (BOD) values monthly compared with the seasonal olive oil production capacities may be done in the future studies to better characterize the potential toxicity effect of OMWW on the lake.

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