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Olive mill wastewater: Effect on the survival and reproduction of the ecological indicator "*Gammarus gauthieri*"

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Abstract
Olive oil production is an important agricultural activity in Morocco, which constitutes an important source of revenue for farmers. However, the olive oil industry generates large amounts of olive mill wastewater (OMW). The OMW disposal poses significant environmental problems. The objective of this study is to evaluate the effect of OMW on the environment as indicated by the survival and reproduction of the aquatic bio-indicator *Gammarus gauthieri*. The first experiment was conducted to test the effect of different concentrations of OMW, ranging from 1 to 16 ml of OMW / 250 ml, of lake water on *Gammarus gauthieri* survival. The results obtained showed a high toxicity of OMW, with TL_{99} ranging from 0.99 to 1.71 days. The second experiment tested the effect OMW at a concentration of 0.25 ml / 250ml of lake water on the reproduction rate of *Gammarus gauthieri* over time. The results obtained showed a remarkable effect of the OMW. Compared to the control. OMW presented a stimulating effect on the reproduction of *G. gauthieri* individuals during the first two days. Then, the reproduction rate decreased until it became zero. The improper management of OMW is a potential threat for the *G. gauthieri* and therefore this might impact the ecosystem equilibrium.

Keywords: Olive mill wastewater, *G. Gauthieri* survival, reproduction rate.

1. Introduction
The olive tree (*Olea europaea*) is one of the oldest oil trees in the world. Olive oil production is mainly concentrated in the Mediterranean region, where olive oil activity is a heritage that has lasted for centuries [1] furthermore, olive oil production plays a important role to in boosting farmers' income.Worldwide, the olive oil industry generates large amounts of olive mill waste water "OMW" [2], amounting to $7*10^6$- $30*10^6$ m^3 [3]. In Morocco, the annual OMW production exceeds 250 000 m^3 [4].
The OMW is a polluter of the environment. Although the amount of OWM produced is significantly small compared to other types of industrial wastes and olive oil production is a seasonal activity, the contribution of OMW to environmental pollution is not negligible. In fact, 1 m^3 of OMW is reported to be equivalent to 200m^3 of domestic waste water [2]. Thus, the OMW polluting impact is an environmental concern that needs to be addressed [2].
To evaluate the effect of OMW on the environment and particularly on the aquatic wildlife, it appeared appropriate to test OMW effect on an ecological bio-indicator *Gammarus gauthieri* (S. Karaman, 1935) (Amphipoda, Gammaridae). Scientists consider this species as one of the most used organisms in eco-toxicological experiments. *G. gauthieri* is considered as an indicator in the assessment of pollution hazards at the water level [5]. Indeed, *G. gauthieri* is largely abundant in the aquatic media in Morocco, living at high density and easy to identify. It is also known for its sensitivity to a wide range of chemical products and plays a major

role in the decomposition of animal and vegetal litter, thus, impacting the food chain. *G. gauthieri* is also considered a prey for many fish species [5, 6, 7, 8]. These the reasons were behind our choice *G. gauthieri*, as an ecological bio-indicator to assess the environmental polluting effect of OMW.

2. Experiments description

2.1. Gammarus gauthieri

Gammarus gauthieri adults were collected from the lake 'Ain Vittel' which is located about 4km from the city of Ifrane, Morocco (33°32' North 5°06' West). Six hundred *Gammarus gauthieri* adults were collected using a sieve of a mesh 0.15cm in diameter, and immediately. transported to the laboratory in plastic boxes with water and algae fragments from the lake. Then, the individual *G. gauthieri* adults were maintained at a temperature of 15 °C in the laboratory.

2.2. Olive mill waste water samples

The OMW samples were collected on November 2014 from a traditional olive oil production unit, located in the city of Sefrou, Morocco (33°49'50" North, 4°50'15" West). Sampling was performed immediately after olive oil extraction and OMW production, at three times with an interval of 15 min between each sample collection. The collected samples were stored in closed boxes at 4°C to avoid any alteration of their chemical composition.

2.3. Physico-chemical composition of OMW

The OMW analysis (pH, electrical conductivity or EC, levels of crude protein, potassium, phosphorus, magnesium, calcium, copper, manganese, iron, zinc, sodium, cadmium and nickel) was conducted at the official research laboratory of chemical analyses located in Casablanca (www.loarc.ma), Morocco. The rate of solids in suspension, chemical oxygen demand (COD), biological oxygen demand (BOD), nitrite concentration, ammonium, sulfate, nitrate, orthophosphate and chlorine were analyzed as cited by RODIER [9].

2.4. Total polyphenol content

Total polyphenols content of the OMW (expressed in milligrams Gallic acid equivalent per gram of OMW) was determined using the Folin-Ciocalteu method [10]. 200µl of OMW was mixed with 1ml of a freshly prepared reagent of Folin-Ciocalteu (10 times diluted) and 0.8ml of sodium carbonate 7.5% (Na_2CO_3). The whole mixture was incubated for 30 minutes at room temperature (20°C). Reading was performed using a spectrophotometer at a wavelength of 765nm.

2.5. Description of experiments

2.5.1. Experiment 1

Bio-tests were designed to study the effect of different concentrations of OMW on *G. gauthieri* survival. the 10 *G. gauthieri* individuals ranging from 6 to 8 mm long were put in a plastic box containing 250 ml of lake water mixed with different volumes of the OMW (0; 1; 2; 4; 8 and 16 ml). The boxes were manufactured to allow for air circulation. The boxes were stored at a temperature of 20 °C and a photoperiod of 14 hours of light and 10 hours of darkness. Evaluation of *G. gauthieri* survival was monitored daily. Survived individuals are counted and dead individuals removed [7] . Survival rate tracking was conducted till the death of all *G. gauthieri* individuals. Every *G. gauthieri* individual unable to move and respond to a mechanical stimulus was considered dead. The experiment was conducted as a completely randomized design (CRD). The experimental unit consisted of a 0.5l plastic box containing ten *G. gauthieri* individuals in 250ml of lake water. Each OMW concentration used was repeated five times.

The measurement of the toxicity of OMW was done by calculating the observed mortality rates. Survival curves were established using Excel software version 2007. The comparison of survival curves was done by the log-rank test [11]. The time required to kill 50% (TL_{50}) or 99% (TL_{99}) of *G. gauthieri* population was determined from the survival curves obtained (time of exposition vs probability of survival).

2.5.2. Experiment 2

The effect of *G. gauthieri* on reproduction was evaluated by mixing 0.25ml of OMW with 250ml of lake water, and 5 *G. gauthieri* adults were introduced into the plastic box. After fecundation of the female by the male, eggs were conserved in the female abdomen till oviposition. The eggs develops to small gammarus larvae similar to adults. The larvae were counted every dayand placed in boxes containing drinking water and nutrients.
The experimental design used is a split plot in time, with days as the main plots and treatments as sub plots were replicated in a CRD. Replication-days were combined and classified as a random effect (i.e., error term) to reduce variability in the fixed effects. Statistical analyses were performed using Analysis of variance (ANOVA) procedure of JMP PRO 12.0 software (SAS Institute, Inc., Cary, NC) and Tukey's HSD test was used for means separation when treatment revealed significant.

3. Results and discussion

3.1. Physico-chemical composition of OMW

The analysis of the composition of OMW is crucial to further assess properties of OMW (Table 1). The OMW has a reddish black color (10R 2.5/1 in the Munsell code), with an acidic liquid of high EC, which was generated during olive oil production [12]. In general, OMW samples are slightly acidic; the pH depends on the type of olive oil processing. The pH of OMW from pressure processing system varies between 4.5 and 5, while pH of OMW from centrifugation processing system ranges between 4.7 and 5.2 [12].
The OMW from the semi-modern unit showed high electrical conductivity (43mS.cm^{-1}), which was three times more (13±0.5mS.cm^{-1}) than samples of traditional processing unit (13.5 mS.cm^{-1}) [2]. The composition of OMW is subject to variation, depending on the olive tree variety, soil type, wheather conditions, crop production systems, and the olive oil extraction method [13, 14, 15].

Table 1: Physico-chemical properties of OMW collected on November 2014, from a traditional processing unit, located in the city of Sefrou, Morocco

Parameters	OMW
pH	5.0
EC (mS.cm^{-1})	13.5
SM (g.l^{-1})	40
COD (g.l^{-1})	160
BOD_5(g.l^{-1})	90
Crude protein (%)	1.4
Potassium (%)	1.0
Phosphorus (mg.kg^{-1})	641.8
Magnesium (%)	0.045
Calcium (%)	0.1
Copper (mg.kg^{-1})	2.2
Manganese (mg.kg^{-1})	4.5
Iron (mg.kg^{-1})	40.0
Zinc (mg.kg^{-1})	10.0
Sodium (%)	0.06
Cadmium (mg.kg^{-1})	< 0.015
Nickel (mg.kg^{-1})	< 0.75
Nitrite (mg.l^{-1})	260.57
Ammonium (mg.l^{-1})	1342.53
Sulfate (mg.l^{-1})	1591.84
Nitrate (mg.l^{-1})	743.39
Orthophosphate (mg.l^{-1})	12.43
Chlorine (g.l^{-1})	184.6

The COD is subject to change depending on the olive oil extraction method. The COD of OMW issued from semi-modern and modern three-phase oil extraction process was 113±9.8 and 51±7.6 (g of $O_2.l^{-1}$), respectively [2]. Also, the concentration of BOD_5, was 90 to 20 (g of $O2.l^{-1}$) of OMW issued from pressure processing system and centrifugation system, respectively [12].

3.2. Total polyphenol content

Previous studies identified that OMW possesses anti-microbial activities and phytotoxic properties, which were attributed mainly to its poly-phenols content [16]. The content of total polyphenols in the OMW was 66.19 mg Gallic acid equivalent / g of OMW. This value is much greater than that cited in the literature, (27.5±3.5mgl^{-1}Gallic acid equivalent) collected from a three-phase olive processing unit [3]. Because of the high poly-phenols content in the OMW, their disposal into water reservoirs was prohibited in some countries of the European Union [16].

3.3. Effect of OMW on the survival of G. gauthieri adults

The application of OMW showed significant toxicity and affects the survival of *G. gauthieri*. The survival rate of *G. gauthieri* individuals depend on the concentration and the duration of exposure to OMW. The probability of survival of individuals in the control treatment was greater than all other treatments. At the treated units, all concentrations caused mortality of all individuals in only 1-2 days (Fig. 1).

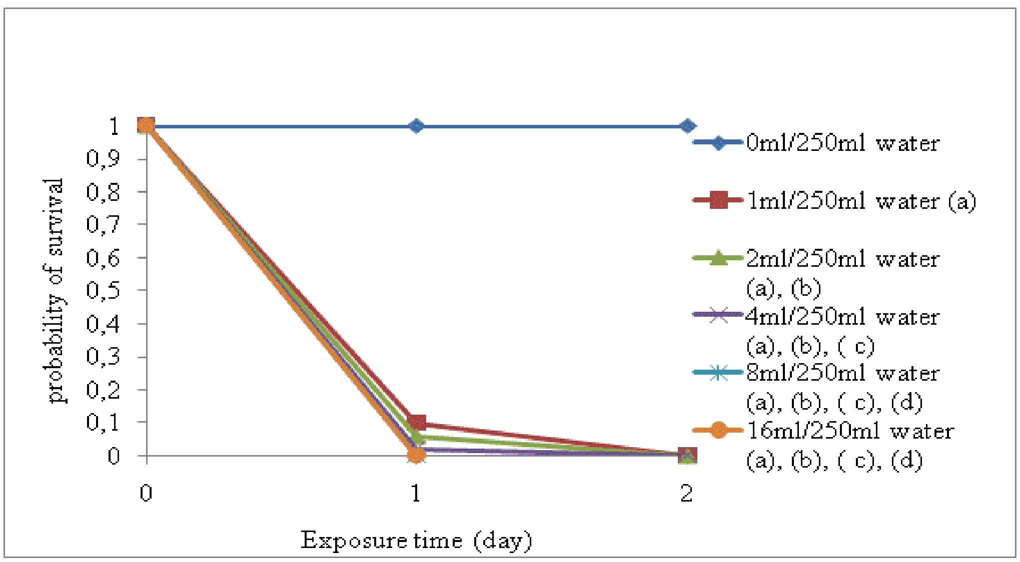

Figure 1: *G. Gauthier* survival curves treated by OMW (concentrations are in ml of OMW / 250 ml of lake water Ain Vittel) (The same letters indicate no significant difference at a probability level of 5%).

Second, the TL_{50} and TL_{99} (time required to kill 50% and 99% of the treated *G. gauthieri* population) are negatively correlated to the exposure time. The coefficient of correlation approaches −1 which explain the stronger correlation between concentrations of OMW (ml / 250 ml water AinVittel) and TL50/ TL99 of G. gauthieri. The TL_{50}increased from 0.5 day for concentration equals to 16ml of OMW / 250 ml of water to 0.73 day for aconcentration equals to 1ml of OMW / 250 ml (Table 2).
Indeed, the addition of OMW in water tanks without pre-treatment, led to serious problems for the whole eco-system [2]. It was reported that *Mytilus galloprovincialis* exposure to concentrations of OMW from 0.01 to 0.1% caused pre-pathological alterations in their molds tissue [17]. Furthermore, studies showed that treating seven strains of oyster with concentrations of OMW ranging from 0 to 90%, affected the development of oysters,

decreasing their reproduction, and their ability to move [18]. Our study was the first of its kind to test the negative impacts of OMW on *G. gauthieri*.

Table 2: Relationship between concentrations of OMW (ml / 250 ml water AinVittel) and TL_{50}/ TL_{99} of *G. gauthieri*

OMW	TL_{50} (days)	r*	TL_{99} (days)	r
0	-		-	
1	0,73		1,71	
2	0,71		1,69	
4	0,68	-0,88	1,66	-0.88
8	0,50		0,99	
16	0,50		0,99	

* : r (r_{th} = 0,71, dl=4, α=0.05)

3.4. Effects of OMW on the reproduction of G.gauthieri

Prolonged exposure to low concentrations of OMW may lead to sublethal effects, including changes in the rate of fecundity of some species. Exposure of *G. gauthieri* individuals to OMW triggered remarkable effects on their reproduction. Compared to the control, OMW stimulated the reproduction of individuals in the first two days. Around seven larvae were born the first day. Afterwards, the reproduction rate decreased systematically till zero. The untreated units showed a variation in fecundity rates over time (Fig. 2).

The analysis of variance showed a significant difference between the treated and the untreated experimental units at the first and the fifth day of the experiment. This difference can be attributed to the activity of poly-phenols in the OMW. A study conducted on the effect of tannic acid on the fertility of mice showed that tannic acid interferes with the neuro-secretion activity of the hypothalamus, which directly impact the hormonal regulation of the reproductive cycle of mice [19].

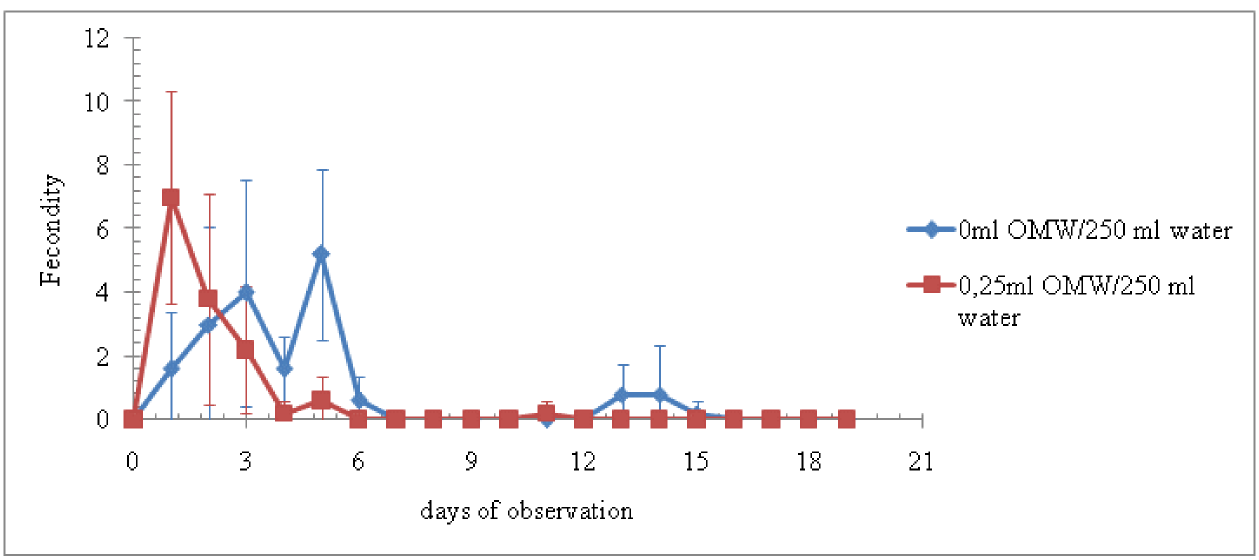

Figure 2: Variation curves of fecundity of *G. gauthieri* treated with the OMW.

As the concentrations of OWM at the water level (ground water reservoirs, surface aquatic reservoirs, seashores, and sea) were unknown, the continuous disposal of these effluents into aquatic zones causes lethal toxicities and threatens the existence of many species. Also, OMW might induce sub-lethal effects, including behavior changes such as a reduction in the rate of predation. This might create abnormalities in the diet of *G. gauthieri* individuals, and impact negatively their growth potential [20].
OMW was claimed to be one of the most polluting effluents produced by the industry since it has a high polluting load. The physico-chemical characteristics of OMW, which are related directly to its acidic pH, high EC, high organic matter and poly-phenols content, high biological and chemical oxygen demand [21] might cause toxicity towards plants, bacteria, and aquatic organisms [22].
Polyphenols were composed of a wide range of chemical products mainly Hydroxytyrosol, Tyrosol, caffeic acid, protocatechuic acid, p-coumaric acid, para-hydroxyphenylacetic acid, Verbascosid, Luteolin, the elenolic acid, apigenin, Ligstrosid, oleuropein, Rutin and hydroxytyrosol which was the most abundant [22, 23, 24].
On the other hand, the heavy metals present in the OMW were also liable for the toxicity of effluents especially when the concentration of OMW in the effluent exceeds a threshold. The penetration of metals into the body of *G. gauthieri* was performed in three ways: (1) by the absorption of food and water; (2) through the cuticle or (3) via the exchange surfaces (gills). Generally and in many freshwater species, metals were concentrated in the gills where they can significantly influence osmo-regulation and respiration processes [25]. Particularly, cadmium which affects the physiology and the behavior of *G. gauthieri*, by causing a significant decrease of the osmolality and the concentration of Ca in their tissues. Behavioral responses such as the diet, and the ability to move might also significantly change in *G. Gauthieri* [26].

Conclusions

The OMW were waste products resulting from the production of olive oil. Their disposal causes a serious environmental problem of major concern. To eliminate or the alleviate effects of OMW, advanced technologies can be addressed. Some of these techniques do not enable to suppress the polluting power of OMW, while other techniques were expensive or generate side effects, requiring subsequent processing [27]. As a result, there was no approved method to treat OMW, because of technical and economic limitations [28]. The potential adequate solution for this problem was to use these effluents for the production of bio-fuel [29] and compost [30-32].

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