Effect of polyphenols on activated sludge biomass during the treatment of highly diluted olive mill wastewaters: biomass dynamics and purifying performances

T. E. Elmansour, L. Mandi, A. Ahmali, A. Elghadraoui, F. Aziz, A. Hejjaj, M. Del Bubba and N. Ouazzani

ABSTRACT

This study aims to investigate the feasibility of treating olive mill waste water (OMWW) by activated sludge pilot (AS) after its high dilution (1%) by urban waste water (UWW) and to study the effect of polyphenol compounds on the biomass during the treatment. Specific oxygen uptake rate (SOUR), mixed liquor volatile suspended solids (MLVSS), chemical oxygen demand (COD) and total polyphenols, were followed up over 100 days. In spite of the polyphenols' high concentration (up to 128 mg·L^{-1}), successful biomass growth of 7.12 g $_{MLVSS}$.L $^{-1}$ and activity were achieved. Most of the bacteria (*Pseudomonas* sp., *Klebsiella oxytoca, Citrobacter fereundii, Escherichia coli* and *Staphylococcus* sp.) and fungi (*Trichoderma* sp., *Rhizopus* sp., *Aspergillus niger*, *Penicillium* sp., *Fusarium* sp., *Alternaria*) identified in the aerobic basin during the stabilization stage were known to be resistant to OMWW and showed effective adaptation of the biomass to polyphenols in high concentration. COD and polyphenols were highly eliminated (90%, 92% respectively). The sludge volume index in the pilot settling tank was almost constant at around 120 mL.g $^{-1}$. This suggests the possibility of managing OMWW by simple injection at a given percentage in already functioning conventional AS treating UWW.

Key words | activated sludge reactor, diluted olive mill wastewater, microbial population, phenolic compounds, treatment efficiency, urban wastewater

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HIGHLIGHTS

- Activated sludge (AS) treating diluted olive mill wastewater by urban WW was tested.
- Biomass grow successfully in spite of high amount of toxic polyphenolic PP compounds.
- Most of identified biomass (bacteria, mold, fungi) are adapted and resistant to PP.
- COD and polyphenols, including toxic fractions, were highly eliminated (90%, 92%).
- We can suggest injecting successfully a % of OMWW in conventional functioning AS.

INTRODUCTION

Olive oil production leads to the generation of a liquid waste by-product, widely known as Olive Mill Wastewater (OMWW). It is produced annually in huge quantities of approximately 5.4 10^6 tons worldwide by olive oil-producing countries. The production of olive oil is concentrated around the Mediterranean basin (about 95% of olive oil production worldwide) (Fiorentino *et al.* 2003; IOC 2017).

OMWW presents antimicrobial activity due mainly to the significant presence of a high load of phenolic compounds, and so can inevitably cause serious environmental hazards when it is released into natural bodies without proper treatment (Meftah *et al.* 2019).

Several approaches have been investigated for OMWW treatment, which apply physico-chemical and biological processes as well as their combinations (Paraskeva & Diamadopoulos 2006; Achak *et al.* 2014, 2009a, 2009b; Hanafi *et al.* 2010; Tekerlekopoulou *et al.* 2017; Oz *et al.* 2018). Physico-chemical techniques have been tested but

doi: 10.2166/wst.2020.423

are generally cost prohibitive, do not yield full purification and are non-environmentally friendly, since they always generate a high amount of other toxic byproducts (Nazari *et al.* 2007).

Otherwise, anaerobic digestion is the most widely adopted for OMWW treatment (Beccari *et al.* 1996). However, anaerobic digestion of OMWW is still hampered and limited, due to the actions exerted on methanogenic bacteria by simple phenolic compounds and certain organic acids (Hamdi 1996).

On the other hand, aerobic biological processes have gained attention over recent years as they permit high possibility to remove various kinds of organic matters and inorganic nutrients in an eco-friendly and relatively cost-effective manner (Moussavi *et al.* 2014). However, aerobic biological approaches for OMWW treatment are still limited by the proper toxicity of polyphenols that causes inhibition of the microbial development and growth even at concentration lower than 200 mg L^{-1} (Marrot *et al.* 2006).

Several works have dealt with aerobic treatments of OMWW testing at laboratory scale separately different microbial communities (Bacteria, fungi, yeast) to reduce the organic load and remove phenolic compounds (Dias *et al.* 2004; McNamara *et al.* 2008; Krastanov *et al.* 2013).

Many different bacteria, coming from activated sludge, have been tested in aerobic processes to OMWW depuration and yielded successful purification regarding chemical oxygen demand (COD) and polyphenol compounds (Borja *et al.* 1995; Benitez *et al.* 1997; Annadurai *et al.* 2002; Trimbake *et al.* 2014; Jaouad *et al.* 2015).

On the other hand, fungi or yeasts have been proposed also to biological treatment of OMWW (García *et al.* 2000). For instance, a white-rot fungi was the most genus used for OMWW remediation and prove effectiveness not only at removing COD and phenolic compounds, but also at reducing coloration. Likewise, *Aspergillus niger* was also reported by many research papers due to its ability to degrade OMWW (Aissam 2003; Mishra & Kumar 2017).

Until recently, the study of wastewater treatment processes by biological approaches was restricted to monitoring the physico-chemical parameters in the influent and effluent, overlooking the biological basin and considering it as a 'black box' where un-identified microorganisms survive. Moreover, the process performance has been generally evaluated without considering the optimal operating conditions for the treatment.

In addition, to the best of our knowledge, there are few works on dynamic researches related to the treatment feasibility of combining real UWW and OMWW by aerobic approaches, and there is lack of information about the exact effect of polyphenols compounds (up to 128 mg·L^{-1}) on the growth, physical properties and physiological state of the microbial consortium involved in the treatment. Excepting some works done by El Moussaoui *et al.* (2017); Jaouad *et al.* (2018a) doing similar aspects, but the concentration of total polyphenols in the mixture composition tested did not exceed 44 mg·L^{-1}. Moreover, these works did not identify in detail the microbial consortium profile involved in the aerated tank biomass.

Starting from the fact that microorganisms in activated sludge are the major component of the treatment system, (Nielsen *et al.* 2004), the current study aims to determine the effect of high concentration of polyphenols components (up to 128 mg·L^{-1}) on the biomass growth and diversity during the treatment of a diluted OMWW by urban wastewater in an activated sludge pilot. Microorganisms physiological state, biomass physical characteristics, microorganisms communities inside the aerobic bioreactor identification and polyphenols removal have been particularly emphasized.

MATERIAL AND METHODS

Reactor setup

A pilot-scale automated activated sludge (AS) plant (TEA/3000) was used in the experiments (see Figure S1 in supplementary material).

The start-up of the pilot plant was conducted using activated sludge from the aeration basin of the wastewater treatment plant of Marrakech city (Morocco).

Biomass samples (2.20 g·L^{-1} of MLSS) were incorporated to the laboratory scale activated sludge, and maintained without aeration and alimentation for one day. After, at the first stage it was fed by the mixture (UWW + OMWW) with 0.1% v/v of OMWW for a period of 20 days, as an acclimation procedure, before increasing the percent volume of OMWW to 1% at the second stage.

UWW and OMWW characterization

Urban wastewater (UWW) used in this study was collected after the primary settling tank of the wastewater treatment plant of Marrakech city (Morocco); their principal characteristics are listed in Table 1.

The OMWW used in this experiment was sampled from a traditional extraction mill of olive oil at 50 km away from the city of Marrakech. Table 1 shows physicochemical and

Table 1 | Physico-chemical and microbiological characteristics (*Mean ± Standard Deviation*) of the different wastewaters used in this experiment

Parameters	UWW (mean± SD)	OMWW	Diluted OMWW
pH (25 °C)	7,07 ± 0,03	5,01 ± 0,04	7,26 ± 0,02
EC (25 °C) ($mS.cm^{-1}$)	4,36 ± 0,25	28,23 ± 0,98	4,44± 0,30
TSS ($mg\ L^{-1}$)	519,33+ 9,45	2,066 ± 11	578 ± 14
COD* ($g\ L^{-1}$)	0,540 ± 0,031	264,05± 11,49	6,10 ± 0,542
BOD_5* ($mg\ L^{-1}$)	318,42 ± 23,72	–	810 ± 1,47
NH_4^+ ($mg\ L^{-1}$)	24,96± 0,06	6,33± 0,30	12,4 ± 0,94
NO_2^- ($mg\ L^{-1}$)	0,04 ± 0,01	96,23± 9,41	2,04 ± 0,084
NTK ($mg\ L^{-1}$)	64,65 ± 0,81	806,66 ± 20,21	200 ± 15
PO_4^{3-} ($mg\ L^{-1}$)	1,44 ± 0,11	31,14± 0,65	9,45 ± 0,46
Total phosphorus ($mg\ L^{-1}$)	1,95 ± 0,06	41,61± 4,37	10,19 ± 0,48
Total phenols ($g\ L^{-1}$)	0.04 ± 0,002	8,73 ± 0,43	0,128 ± 0,003
K^+ (ppm)	406 ± 1	582 ± 5	480,4
Ca^{2+} (ppm)	2,452 ± 40	1,707 ± 15	257,5 ± 12
Total coliform ($CFU^*.mL^{-1}$)	(8,67 + 0,15) $x10^6$	0	(5,30± 0,4).10^6
Fecal coliform ($CFU^*.mL^{-1}$)	(2,13 ± 0,48) $x10^7$	0	(3,70 ± 0,5)10^6
Streptococcus ($CFU^*.mL^{-1}$)	(1± 0,05)$x10^3$	0	(1,27± 0,05)10^5

Note: COD: chemical oxygen demand; BOD_5: biological oxygen demand; CFU: colony-forming units.

microbiological characteristic of OMWW, UWW and the diluted OMWW (1%) used in this experiment. The OMWW is characterized by a high degree of organic matter content (COD) up to 275 $g{\cdot}L^{-1}$, high electric conductivity (28.23 $mS{\cdot}cm^{-1}$), a high content of phenolic compounds (8.7 $g{\cdot}L^{-1}$) and a mildly acidic pH (5.01).

The main characteristics of the diluted OMWW are described in the Table 1. The majority of the influent harsh parameters (COD, EC, and pH) have been fixed with the dilution. However, even if the concentration of polyphenols decreased to 128 mg/L but still enough higher to the biomass bearable concentration. The dilution with municipal wastewater is supposed to provide also a large fraction of microorganisms that could play an important role in the treatment in the biological reactor.

Experimental setup and operating parameters

The activated sludge reactor was continuously operating in order to monitor biomass adaptation and growth as well as system efficiency in terms of COD_T and polyphenols elimination.

The operating parameters of the activated sludge (AS) pilot unit are summarized in Table 2.

Table 2 | Operating parameters in pilot unit

Parameters	A.S pilot
HRT* (d)	1.4 – 1 – 0.7
SRT* (d)	Infinite – 35
Flux ($L.h^{-1}$)	0.9–2.1
F/M* ratio ($kg_{COD}.kg^{-1}_{MLVSS}.d^{-1}$)	0.1–0.3
pH level	7–8
Dissolved oxygen level ($mg{\cdot}L^{-1}$)	2–3

Note: HRT* (hydraulic retention time), SRT* (sludge retention time), F/M* (food to microorganism).

Analytical techniques

Physicochemical parameters, COD, biological oxygen demand (BOD_5), total suspended solids (TSS), mixed liquor suspended solids (MLSS), mixed liquor volatile suspended solids (MLVSS), Nitrite (NO_2^-), Nitrate (NO_3^-), Ammonium (NH_4^+), Orthophosphates (PO_4^{3-}), Calcium (Ca^{2+}), Magnesium (Mg^{2+}), Potassium (K^+) and sludge volume index (SVI) were characterized by standard methods (AFNOR 1997; Rodier *et al.* 2009; APHA (American Public Health Association) 1998).

The total phenolic compounds were dosed according to Folin-Ciocalteau's method (Macheix *et al.* 1990).

Phenolic extracts, were analyzed by high performance liquid chromotography (HPLC) performed using Eurospher II 100-5 C-18 reversed phase column (Knauer-HPLC). All measurements were performed in triplicate.

All the microbial parameters were analyzed according to Moroccan standards methods (Moroccan Standards 2006) and at the stable stage.

The oxygen uptake rate measurement (OUR) ($mgO_2 \cdot L^{-1} \cdot h^{-1}$), was performed in-situ in the bioreactor using oxygen probe (Oxymax COS61/COS61D). The specific oxygen uptake rate (SOUR) ($mgO_2 \cdot g^{-1}_{MLVSS}\ h^{-1}$), was obtained by dividing the OUR by the MLVSS.

RESULTS AND DISCUSSION

The biomass evolution

The adaption of the biomass to the diluted OMWW was estimated by MLSS or (MLVSS). The ratio MLVSS/MLSS was also calculated and shown in Figure 1.

During the application of 0.1% diluted OMWW, the MLVSS was slightly increased before being stabilized (Figure 1). This could mean that the biomass enters into a lag phase, characteristic of an acclimation period to the new substrate composition.

Over stage 2, the biomass concentration remained almost stable until day 29, when it started increasing again to reach 7.12 $g_{MLVSS}.L^{-1}$.

In fact, from day 9 to day 27 the slow growth rate of the biomass, reflected by an almost unchanged MLVSS concentration, indicated that the microbial community was under extremely stressful conditions, and therefore all the energy produced by the cellular metabolism is diverted to manage stress rather than growth. This finding substantiated by the predominant endogenous respiration over this period with an average $SOUR_{inhibition}$ value of 62.83% as indicated in Figure 2.

There was a negative correlation between the MLVSS and the $SOUR_{inhibition}$ ($r = -0.83$, $p = 0.006$). The excess of sludge was extracted from the clarifier, to comply with the pilot's operating parameters. This operation was done considering an SRT of 35 days.

After this operation, a substantial drop in biomass was noticed and it reached an average of $4.78 \pm 0.88\ gL^{-1}$ MLVSS.

Indeed, the relatively high biomass concentration induces an increase in viscosity of the mixed liquor, which affects the oxygen transfer in the liquid phase, leading to a reduction of the aeration rates of the microorganisms and thus to lower purification efficiency (Cornel *et al.* 2003; Marrot *et al.* 2005; Vaxelaire *et al.* 2010).

In addition, the ratio MLVSS/MLSS was constant, $(75 \pm 2)\%$, during the whole experiment, indicating that

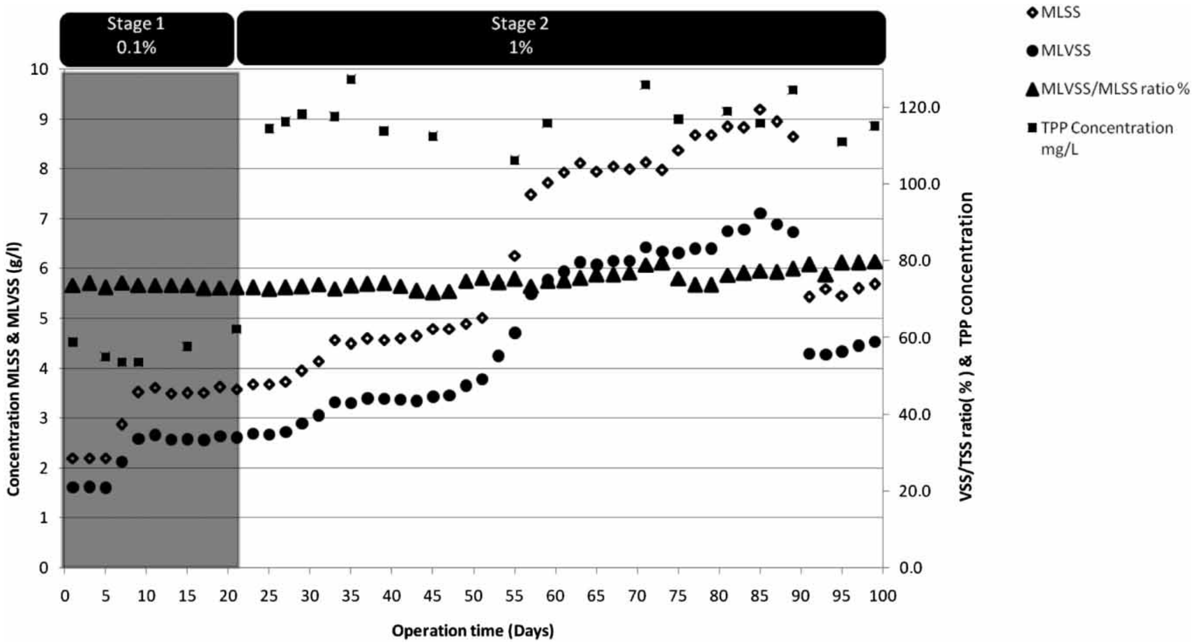

Figure 1 | Biomass growth estimated by MLSS, MLVSS, MLVSS/MLSS ratio and TPP concentration over the experiment.

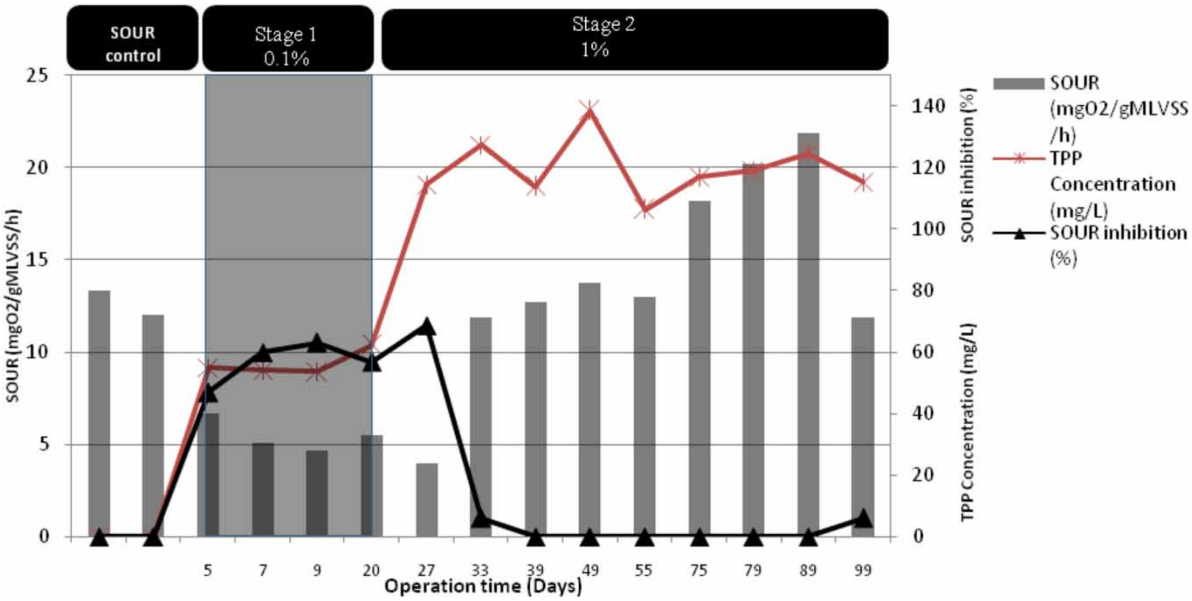

Figure 2 | SOUR and $SOUR_{inhibition}$ versus increasing TPP concentration over experiment time.

organic matters in the biomass community are dominant (no mineralization occurred over time).

The presence of a considerable concentration of biomass in the biological basin remains a good indicator of the perfect adaptation of microorganisms capable of effectively treating diluted olive mill wastewater in UWW.

Microbial activity measurement

The biological respiratory activity was monitored during the experiments (Figure 2). The SOUR evolution over the treatment was variable, with a strong increase in endogenous respiration immediately after the beginning of the experiment. Indeed, the endogenous respiration was predominant during the 27 first days of the treatment with a SOUR average value of 5.1 ± 1.02 $mg_{O2}.g^{-1}_{MLVSS}.h^{-1}$. The composition of the mixture with both OMWW percentages 0.1 and 1% was not used by the biomass as a substrate to supply their energy demand during this period. On the contrary, the bacteria degrade their intracellular carbon to provide the necessary energy for cell maintenance viability and protection against the OMWW toxicity, and the resulting respiration is at its lowest (Aubenneau *et al.* 2010; Villain & Marrot 2013).

These results corroborate with a study done by Henze *et al.* (2001) which suggests that sludge could be exposed to a non-easily biodegradable substrate or toxic substances when the SOUR values ranged between 5 and 10 $mg_{O2}.g^{-1}_{MLVSS}.h^{-1}$. Furthermore, Gutierrez *et al.* (2002) and Sher *et al.* (2000) confirm that the addition of toxic substances and some organic compounds such as 3,5-dichlorophenol and phenol inhibits the sludge activity.

Değermenci *et al.* (2016) studied the treatment of crude olive mill wastewater in a jet loop membrane bioreactor, reported that a SOUR value smaller than 10 $mg_{O2}.g^{-1}_{MLVSS}.h^{-1}$ might be an indicator of inhibited sludge caused by the very toxic and harsh OMWW.

The obtained SOUR values during this phase of treatment agreed with the observation of El Moussaoui *et al.* (2017).

The OMWW toxicity to biomass is often related to polyphenols. The action exerted by total polyphenols certainly inhibited microbial activity, as confirmed by the results found in this study (Figure 2), which led to floc destabilization and low compactness of sludge, causing low settleability (Yu & Gu 1996; Diez *et al.* 2002).

Indeed, when the biomass activated endogenous respiration under stressful conditions, the residue resulting from endogenous metabolism is mainly formed of cell capsules that contribute to low sludge settling properties (Diez *et al.* 2002).

The change of the physical characteristics of the biomass quantified by the SVI variation, as mentioned in Figure 3 below, showed strongest correlations to exogenous respiration and physiological states of microorganisms, evidenced by SOUR values. In fact, the correlation test confirmed the significant negative relationship between the two metrics with a Pearson rank coefficient equal to -0.845 ($r = -0.845$, $p = 0.008$).

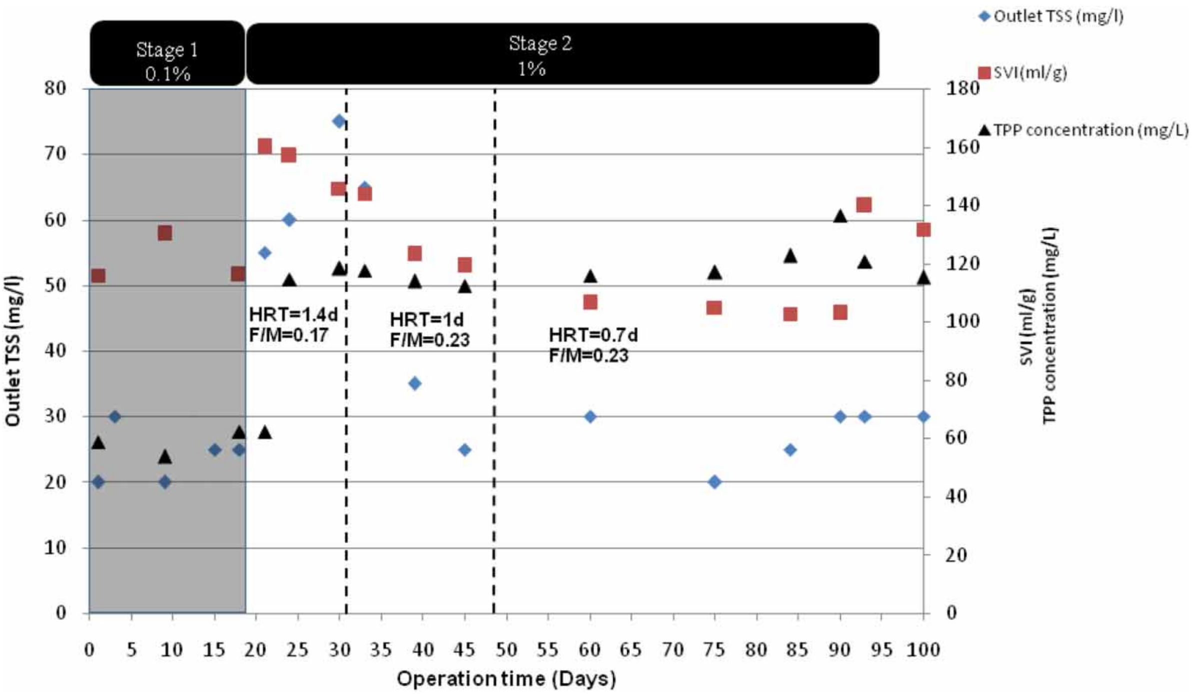

Figure 3 | TSS concentration in outlet and SVI versus TPP concentration during the experiment time.

However, after a few days of the system running, the SOUR value increased significantly with a mean value of 12.83 ± 0.77 $mgO_2.g_{MLVSS}^{-1}.h^{-1}$. The evolution of biomass exogenous activity during this period from day 33 to day 55, with COD removal, always maintained around 90%, indicating that the microbial community starts adapting itself to the system as well as to the toxic and harsh OMWW.

By the end of the experiment, the SOUR value started increasing again and reached a maximal value of 21.88 $mgO_2.g_{MLVSS}^{-1}.h^{-1}$, indicating a satisfactory level of microbial activity within the aerobic basin. This increase could be directly linked to the fact that toxic elements present in OMWW no longer appeared to disrupt biomass respiration, which highlights good adaptability of the biomass to the mixture.

The TSS concentration in the effluent and the SVI are given in Figure 3.

The TSS concentration in the outlet was low on average 24 ± 4.18 $mg_{TSS}.L^{-1}$, during the first stage of the experiment, due to the good sedimentation of flocs, confirmed by a sludge volume index SVI of 120 on average.

From the startup of the second stage until day 33 with an HRT of 1.4 d, the SVI increased drastically to 151.6 ± 8.03, leading to poor sludge coagulation properties, which barely settled in the clarifiers, then the TSS concentration in the outlet became high ($63.75 \pm 8.54 mgL^{-1}$).

This alteration of the flocs' physical characteristics could be attributed to the action exerted by toxic compounds present in OMWW, especially TPP that could prevent flocs from aggregation, which led to low settleability (Yu & Gu 1996).

The physical characteristics of biomass seemed to be proved after changing the HTR to 1 d, where the SVI was found to decrease with an average value of 121.08 $mL.g^{-1}$, and the TSS concentration measured in the effluent stabilized at 30 mgL^{-1}, which proves that the heterogeneous composition of OMWW was no longer appearing to disrupt biomass structure.

During the best stable reactor operation time (55–100th days), (HRT 0.7d) the SVI values scattered around 102 to 131 $mL{\cdot}g^{-1}$ giving good sludge settleability with a clear effluent.

The good sludge sedimentation by the end of the experiment could be ascribable to SRT adopted in this period. According to literature, the good sludge flocculation and SRT follow similar trend lines, the sludge sedimentation improved as the SRT increased (Grady *et al.* 2011; Li *et al.* 2014). In the present study, the flocs' settleability quantified as a mean of SVI and turbidity in outlet effluent showed strongest correlations to SRT, and that was one of the most important key parameters affecting the biomass development and aggregation of biomass into clusters.

Treatment performance of the activated sludge pilot

Figure 4 depicts the COD variation as well as overall removal performance, in the pilot-scale activated sludge, under different HRT and F/M, during the two stages of the experiment..

Results shows that the COD removal was not really affected by the COD feeding concentration except for the period of the beginning of the second phase, with an increase of OMWW volume percentage from 0.1% to 1%, synchronized with a slight decrease in COD removal efficiency to 77.6% ± 1.15. Otherwise, high treatment efficiency greater than 80% was obtained.

Indeed, when influent COD values were at their maximum level, the COD in the effluent did not undergo a noticeable change.

Though the COD removal slightly decreased with the start-up of the second stage, when the bioreactor was operated with HRT of 1.4 d, the total COD removal efficiency of the activated sludge process could be kept over 85% independently of HRT variation. The bioreactor was responsible for 77–86% of COD removal.

Throughout the second stage of the experiment, especially after day 55, the high COD removal was maintained and reached in spite of the increasing OMWW, with an average drop of 92.75% ± 2.13%, indicating a good stabilization of the system under the operating conditions (infinite SRT and SRT = 35d; HRT = 0.7d; Cm = 0.23 $kg_{COD}\ kg_{MLVSS}^{-1}\ day^{-1}$), confirmed by the intense microbiological activity, the reactor's biomass growth, and the successful acclimation of a specialized mixture of microorganisms to this toxic influent, able to effectively oxidize the organic compounds.

Pearson rank correlation coefficient and *p*-values were calculated in order to highlight the relation between COD removal and biomass growth, estimated as MLVSS. Correlation analyses confirmed that the biomass growth influenced the COD removal, and a positive correlation could be established between the two metrics ($r = 0.761$, $p = 0.028$).

The obtained results confirm what was found by El Moussaoui *et al.* (2017) in a conventional activated sludge treating OMWW (COD removal 86%) and those cited by (Jaouad *et al.* 2015) in a membrane bioreactor (up to 90%) indicating that the biomass could well resist a strong load in COD despite the toxic environment. However, Chiavola *et al.* (2014) found a lower COD removal (60%) than ours when treating diluted OMWW by municipal wastewater in a laboratory-scale sequencing batch reactor (SBR). Dhaouadi *et al.* (2008) mention a COD elimination of approximately 80% when an OMWW treated of around 1,500 ppm COD was used, proving that the biomass resisted satisfactory a strong load of COD. Otherwise, according to the same authors, when the biomass was in contact with a

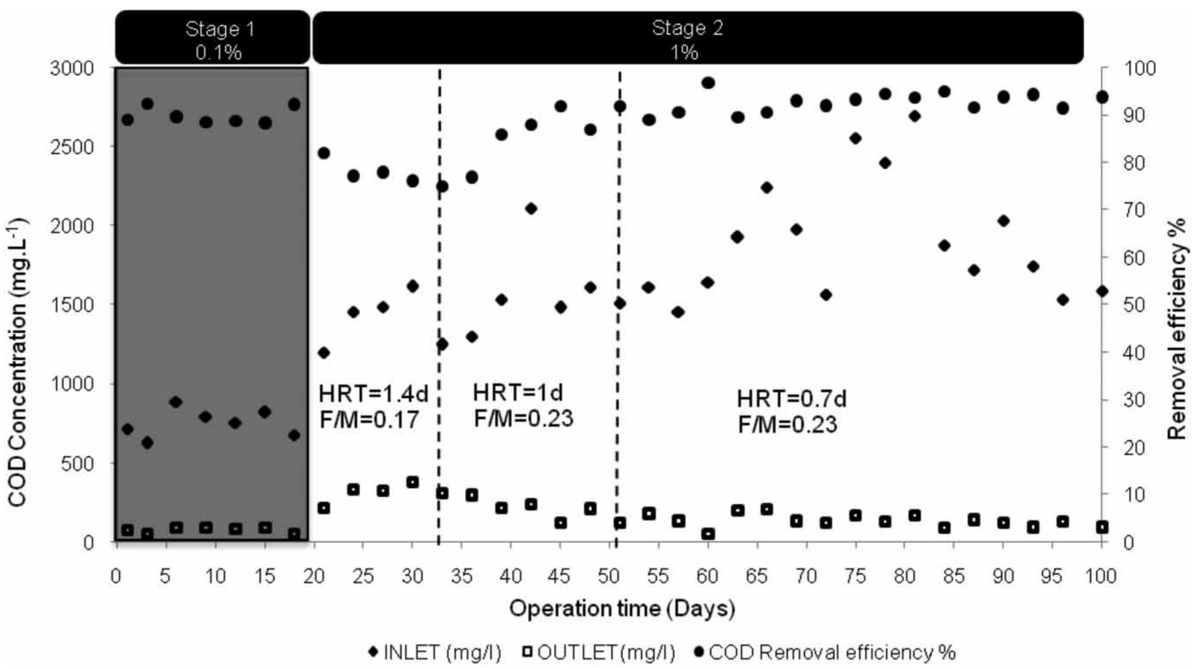

Figure 4 | COD variation and overall removal performance during the two stages of the experiment.

very high concentration of COD (5,300 ppm) it could only eliminate 37%. Değermenci *et al.* (2016) found a COD removal of 92% in an aerobic jet loop membrane bioreactor, independently from the high organic load.

The TPP concentrations in the inlet and outlet of the reactor, as well as the overall removal efficiencies observed at the two stages of the experiment, are plotted in Figure 5.

As can be seen, during the first stage, the TPP removal was very low and it barely reached 29.4 ± 7.83% on average. However, with the beginning of the second stage, at HRT 1.4d, a marked decrease was noted in efficiency, dropping to 19% immediately after increasing the proportion of OMWW in the influent from 0.1% to 1% (concentration of polyphenols from 10 to 128 $mg{\cdot}L^{-1}$). In addition, the period from the startup of the second stage to day 55 was characterized by a slight increase in degradation accounting for 28.33% ± 7.33 on average. After day 55 (HRT = 0.7d, $F/M = 0.23\ kg_{COD}.kg^{-1}_{MLVSS}.d^{-1}$), the rate of TPP removal increased substantially to reach 93% at the end of the experiment.

Moreover, HPLC analysis carried out on influent before and after treatment has confirmed the obtained results and has revealed the removal of hydroxytyrosol and cinnamic acid, the major phenolic fraction present in the feed influent.

According to the previous results, we could conclude that even though the present activated sludge system could be operated with a high performance under lower HRT values, the general assessment of TPP removal appears to be affected by SRT rather than by HRT. In a study performed by Michailides *et al.* (2011) about the biological treatment of OMWW in a full-scale system, phenol oxidation did not seem to be affected by HRT's variation.

The disorder in the biological treatment in terms of TPP removal in the startup of the second stage could be explained by the high concentration of TPP applied within the bioreactor. Based on these results, and starting from the fact that toxicity of OMWW is generally due to polyphenols, it is likely to deem that the established concentration (119.65 ± 9.17 $mg{\cdot}L^{-1}$) inhibited the microbial consortium, which could not withstand the high TPP load.

This finding is analogous to a study done by D'Annibale *et al.* (2004b) investigating the potential of the white-rot fungus *Panus tigrinus* in removing phenols from OMWW. At the beginning of the cycle, the authors found a long lag phase with a low removal efficiency of phenols, in case of high load wastewater.

In fact, this might be explained by the fact that microorganisms that prevail in the aerobic basin supposed to be

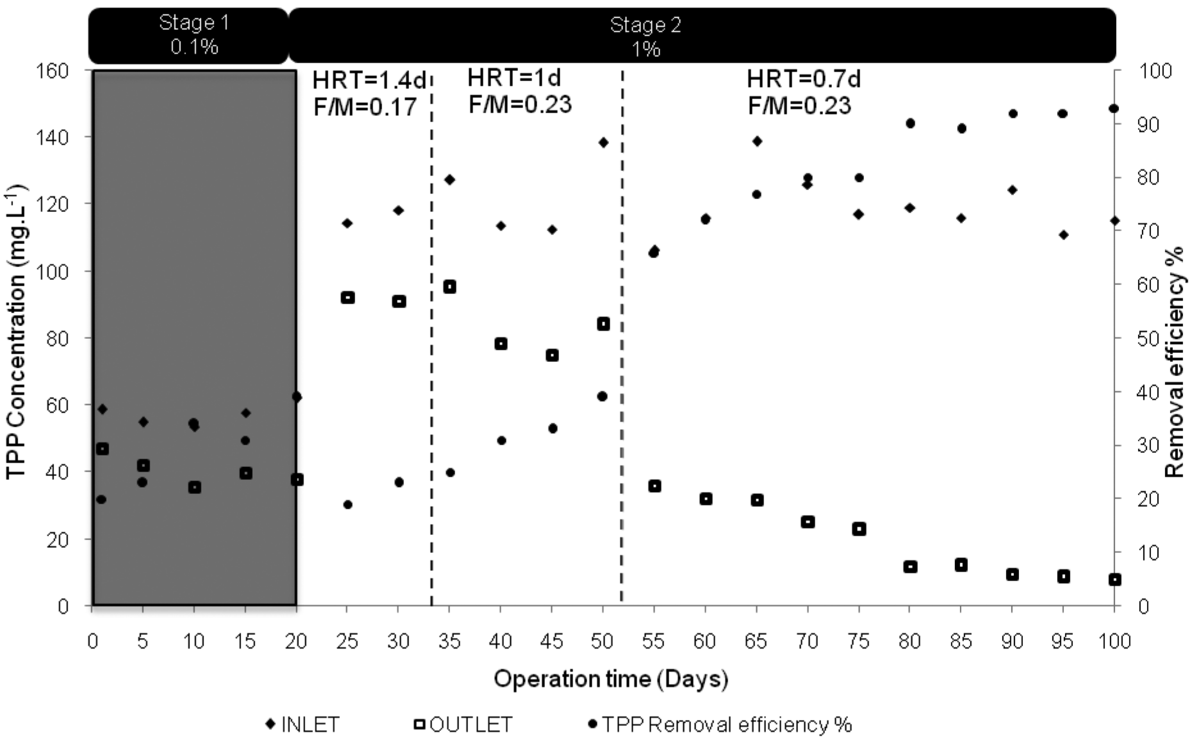

Figure 5 | TPP variations and removal efficiency at different HRTs.

responsible for the TPP degradation are still not well acclimated to the new composition of the influent and thus they are still not possessing the enzymatic material necessary to degrade the phenolic compounds. This explanation seemed to be confirmed by the same authors mentioned above, but in another study (D'Annibale *et al.* 2004a), where they suggest that the lag phase could be imputable to the time needed by the fungus to adapt its mechanisms in order to effectively metabolize the toxic compounds of OMWW.

Indeed, according to literature works (Chung *et al.* 2004; Agarry *et al.* 2008), the TPP degradation is attributed to a specific population of microorganisms that are revealed as the processing goes by. Since the treatment was in its beginning the new population that utilizes the phenols as the sole source of carbon still had not yet developed (Neumann *et al.* 2004; Geng *et al.* 2006; Dhaouadi *et al.* 2008).

By the end of the experiment, the TPP removal remained fairly high and constant. This result could be ascribable to the presence of a specialized mixture of purified biomass inside the aerobic basin, composed of the most appropriate species (bacteria, fungi and yeasts) selected and adapted during the 49 days of the system running. These new populations of microorganisms have been widely reported in much scientific literature to be directly involved in the specific degradation of phenols (Ammar *et al.* 2005; Chaojie *et al.* 2007; Hachicha *et al.* 2008; Krastanov *et al.* 2013). This observation is corroborated by microbial analysis performed in this study, as shown in Table 3 below, which summarized the results of the microbial analysis by the end of the experiment.

Furthermore, to confirm the obtained results, when TPP elimination and biomass growth values are evaluated together, especially after day 49 when biomass sees a noticeable growth rate. It can be concluded that both parameters are scaled linearly, with a Pearson correlation coefficient equal to 0.958 ($r = 0.958$, $p = 0.001$).

Moreover, correlation analyses confirmed that biomass activity influenced the TPP elimination rate. The Pearson rank correlation coefficient ($r = 0.761$, $p = 0.004$) confirmed the positive relationships between the two metrics. Indeed, the intensive microbiological activity was an indication of the TPP degradation, except for the period from day 33 to day 49, where high exogenous respiration was associated with low TPP abatement, which assumes that the most oxygen uptake was used by the biomass to survive and for protection under stressful conditions (average TPP concentration of 119.08 ± 6.87 mg·L^{-1}) rather than for polyphenol elimination.

The obtained results are in line with those of a research related to biomass behavior in an activated sludge process treating OMWW, which indicated that the phenolic compounds removal (93%) was achieved as a result of the biomass adaptability to this toxic effluent (good growth and stable physiological state) (El Moussaoui *et al.* 2017).

Despite the low biodegradation observed at the beginning of the second stage, due to the inhibitory effect of total polyphenols on the microbial metabolism, the conventional activated sludge system gave interesting results by the end of the experiment, compared with ones found in literature in term of TPP removal.

In a study investigating the performance of an activated sludge process for municipal and OMWW treatment, the phenol removal rate has been found to be 80%, with a low concentration applied to the system of total phenols, about 40 mg·L^{-1} (Nesseris & Stasinakis 2012). The study revealed that the SBR (Sequencing Batch Reactor) operated with good performance when it is fed with 1% v/v OMWW; however, the purification yield of the system in terms of COD and total phenols removal was affected when the concentration of OMWW was increased to 5% v/v.

Comparing the results obtained in this work with other ones found in a study performed in a jetloop bioreactor treating OMWW-previously pretreated and diluted with a total phenol concentration between 318 and 483 mg·L^{-1}, it was found that the removal efficiency was within the range of 72.6–92.4% (Değermenci *et al.* 2016).

Another study performed in a jet loop bioreactor treating raw OMWW with an average concentration of 1 g.L^{-1}, conducted by Jail (2010), showed that the aerobic system significantly reduced the polyphenols (up to 68%). According to these authors, the performance was obtained as an action of an acclimated microbial consortium, and the oxygenation conditions of the jetloop reactor aeration system.

Table 3 | Microbial composition of the aerated tank biomass at stage 2

Genus	Average ($n = 5$) of microbial abundance (logCFU/ml)
ARB 22*	6,55 ± 0,47
ARB 37*	7.12 ± 056
Pseudomonas sp.	6.41 ± 1.34
Staphylococci	6.20 ± 0.43
Yeast	6,32 ± 0,36
Mold	5,98 ± 0.52
Total fungi	6,43 ± 0.53

Note: ARB22-ARB37*: Aerobic revivable bacteria respectively at 22 and 37 °C.

The results obtained through the experiment show that the acclimation process realized under specific operating conditions including infinite SRT is of major importance to ensure the retention of optimal enzymatic material synthesized by OMWW-adapted microorganisms involved in TPP biodegradation. Many authors have studied the ability of selected microorganisms to remove a wide variety of these compounds.

For example, Marrot *et al.* (2006) reported that an acclimated mixed culture in an immersed membrane bioreactor could successfully be adapted to phenol removal although in high initial concentrations ranging from 500 to 3,000 mg·L^{-1}.

Moreover, a phenol-degrading bacterium obtained from the activated sludge system showed the ability to completely assimilate phenol present at a concentration of 1,700 mg·L^{-1} (Lü & Fu 2005).

In the same context, the results achieved in this study showed closer TPP removal (80%) compared to another recent study treating OMWW with acclimated biomass in an MBR system (laboratory scale) at a lower concentration of TPP in fed influent (around 70 mg·L^{-1}) than the concentration tested in the present experiment (Jaouad *et al.* 2018b).

Microbial analyses

The microbial analysis was carried out on samples collected from the aerobic reactor, at the stable stage of the experiment (Table 3).

The purpose of this part was highlighting the relationships between the abatement of the investigated parameters mentioned above and the presence of a mixed population of microorganisms containing bacteria, yeast mold and fungi, responsible for the key process of UWW and OMWW treatment.

The high abundance of ARB at 22 and 37 °C outlined the good adaptation of the total microbial community within the aerobic reactor to the mixture UWW + OMWW composition under operational conditions.

Concerning the total fungi and yeast and mold, the density was significant, compared with those found in a study treating OMWW diluted with synthetic substrate (El Moussaoui *et al.* 2017) and reached respectively 6.43 ± 0.53 log CFU/ml; 6.32 ± 0.36 log CFU/ml and 5.98 ± 0.52 log CFU/ml.

The *Pseudomonas* genus reached 6.41 ± 1.34 log CFU/ml.

By analyzing the relationship between COD and polyphenols abatement and the abundance of these strains, a unified understanding can be achieved about the essential role of fungi yeast and mold in effective removal of organic pollutants from OMWW.

There are plenty of scientific works that were conducted on microorganisms directly involved in the metabolization of toxic organic compounds present in OMWW (Robles *et al.* 2000; Aggelis *et al.* 2003; Ammar *et al.* 2005; Yan *et al.* 2007; Krastanov *et al.* 2013).

The identification revealed the presence of *Pseudomonas* sp., *Klebsiella oxytoca*, *Citrobacter fereundii*, *Escherichia coli* and *Staphylococcus* sp.

Concerning fungi, six species were identified: *Trichoderma* sp., *Rhizopus* sp., *Aspergillus niger*, *Penicillium* sp., *Fusarium* sp., and *Alternaria*.

Among the latest isolated species, there are ones that proved to be efficient in OMWW remediation and could use polyphenols as a sole source of nutrients (Millán *et al.* 2000). The same author isolated twelve genera from different OMWW disposal lagoons. Among the fungal members identified were *Alternaria, Aspergillus, Fusarium* and *Penicillium*, the same found in our study. These latter exhibited high ability to survive and grow in undiluted OMWW and showed capacity to deplete its antibacterial activity almost completely.

The results found here were also consistent with previous research, which reported that *Aspergillus, Fusarium, Trichoderma* sp., and *Pseudomonas* are among the microorganisms degrading phenols (Mishra & Kumar 2017).

Furthermore, *Aspergillus niger* was also reported to be involved in phenolic compound degradation (Hamdi *et al.* 1991; Hamdi *et al.* 1992; García *et al.* 2000; Aissam 2003).

Kafilzadeh *et al.* 2010 isolated and identified some degrading phenol bacteria from freshwater lake, and found that *Pseudomonas* sp., *Klebsiella* and *Citrobacter* were among the bacteria that were efficient in phenol removal. According to these authors, *Pseudomonas* sp. was the bacteria that shows great ability to assimilate a wide concentration of phenol (0.8–0.9 g.L^{-1}).

Buitrón *et al.* (1998) studied the biodegradation of phenolic compounds by an acclimated activated sludge (acclimation for 70 days to 40 mg phenols.L^{-1}) in a batch system. The authors found that among the identified bacteria responsible for phenol removal, *Pseudomonas* sp. had the highest specific phenol uptake rate.

Staphylococcus sp. was also reported by many studies to be capable of degrading phenol (Ajaz *et al.* 2004).

The microbial analysis showed the selection of most appropriate species that possess enzyme systems that are responsible for the biodegradation of polyphenols. Fungi, for instance, are widely known by their ability to produce enzymes degrading polyaromatic hydrocarbons, like polyphenols. Regarding bacteria, they possess a different mechanism able to resist a high concentration of phenol, like in the case of increasing the fatty acid composition of their lipid membrane (Keweloh *et al.* 1991) and the changing in protein expression related with the efflux of the phenol from the cell (Randall *et al.* 2007). Resistance of bacteria toward polyphenol compounds is directly related to its degradation. The tolerance could be considered as the key mechanism in polyphenols degradation, which further improves the biological purification yields.

CONCLUSION

The main challenge of this experiment was to investigate the effect of polyphenol compounds (up to 128 $mg{\cdot}L^{-1}$) on the growth, physical properties and physiological state of a microbial consortium involved in the treatment of diluted OMWW in UWW at activated sludge pilot scale, and to assess the treatment efficiency under optimal operational conditions by regular monitoring of COD_T and polyphenols.

Despite the heterogeneous and toxic composition of OMWW, microorganisms involved in the treatment could efficiently tolerate the harsh conditions, clearly shown through the cumulative production of sludge up to 7.12 $gMLVSS.L^{-1}$ at the stabilized stage of the experiment, the high microbial respiration activity that attained 21.88 $mg_{O2}.g_{MLVSS}^{-1}.h^{-1}$ and a stable biomass physical properties giving good settling characteristics of flocs.

Throughout the treatment, as a result of a good acclimation and adaptation of selected microorganisms, high removal efficiencies were achieved for COD_T and polyphenols including hydroxytyrosol, and cinnamic acid.

This reduction in pollution parameters was a good indicator of the effectiveness of feeding the used activated sludge pilot by the mixture (OMWW and UWW) and its treatment under controlled operating conditions. A highly significant correlation has been demonstrated between biomass shape and profile on one side and organic and phenol biodegradation on the other side.

The obtained results suggest the possibility of treating such toxic effluent OMW by simple injection at a determined percentage in already functionning conventional AS treating UWW.

ACKNOWLEDGEMENTS

This work was supported by IRRIGATION Project ERA-NETMED_WATER-13-069. The authors would like to thank the National Center of Studies and Research on Water and Energy (CNEREE), Cadi Ayyad University for the scientific and technical assistance and the National Center for Scientific and Technical Research (CNRST) for financial support (Internship, ref. PBER: Edition 2016, 19UCA2016, File number: 482UCA).

DATA AVAILABILITY STATEMENT

All relevant data are included in the paper or its Supplementary Information.

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First received 20 April 2020; accepted in revised form 24 August 2020. Available online 4 September 2020