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RESEARCH ARTICLE

Behaviour of physicochemical and microbiological characteristics of vertical flow constructed wetland substrate after treating a mixture of urban and olive mill wastewaters

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Abstract

The aim of the current work is to evaluate the effect of a mixture of olive mill wastewater (OMWW) and urban wastewater (UW) on constructed wetland (CW) substrate physicochemical parameters, and to study the abundance and behaviour of microbial community at different depths. In this regard, substrate samples were investigated at three depth levels (0–10 cm, 10–20 cm and 20–30 cm) inside a pilot scale CW treating the mixture. In order to compare the obtained results with the conventional case, a control (CW pilot plant treating only UW) was implemented. Result shows that an increase in electrical conductivity (from 0.134 to 0.222 mS/cm in 0–10 cm and from 0.131 to 0.283 mS/cm in 10–20 cm), total dissolved salts (from 65.45 to 108.67 mg/kg in 0–10cm and from 64.33 to 135.3 mg/kg in 10–20 cm), total organic carbon (from 0.86 to 6.84%), total nitrogen (from 0.1 mg/kg to 0.45, 0.43 and 0.41 mg/kg, in 0–10 cm, 10–20 cm and 20–30 cm respectively) and C/N ratio take place in the substrate after the treatment of the mixture. As for the microbiological parameters, treating the mixture in a CW results in an increase in the yeast and fungi which may optimize the biodegradation of compounds such as polyphenols that are non-easily degraded.

Keywords Constructed wetland · Substrate · Physicochemical characteristics · Microbiological characteristics · Polyphenols · Olive mill wastewater

Introduction

Olive oil production is an important agro-industrial activity especially in Mediterranean countries (Elmansour et al. 2020; Bruzzoniti et al. 2020). The most important olive oil-producing countries are Spain, Italy, Greece and Turkey, followed by Tunisia, Portugal, Morocco and Algeria (Paraskeva and Diamadopoulos 2006). Worldwide production of olive oil was estimated in 2002 of about 2.5 million tons, the majority of which is produced in Mediterranean region (Galanakis 2017). This production is in constant growth responding to a dramatic increasing in olive oil consumption mainly by non-producing countries (Saadi et al. 2007). During the process of olive oil extraction, different by-products are produced such as olive pomace and olive mill wastewater (OMWW). OMWW is generated in considerable volumes during the process of olive oil extraction: over 30 million m^3 per year (Barbera et al. 2013). OMWW characteristics and composition could change depending on the multiple factors such as origin, maturity of the fruit and extraction method (Ben Sassi et al. 2006). However, in general, OMWW is a dark brown effluent, characterized by an acidic pH, a very high electrical conductivity (EC), high organic load content, high content of polyphenols and high concentrations of fats, oils and greases (El Ghadraoui et al. 2020; Galanakis 2017; Stefanakis et al. 2014). OMWW discharge without treatment could affect the environment in its different matrixes. In water, OMWW could cause discolouring rivers and streams, decreasing of dissolved oxygen and eutrophication. In soil, the spreading of OMWW could modify the physical parameters

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including porosity, aggregate stability, water retention and hydraulic conductivity and chemical parameters including pH, EC, total phenols and available N, P and K (Barbera et al. 2013). Decrease in soil pH, increased salinity and high phenol concentrations were observed after OMWW application (Chatzistathis and Koutsos 2017). Many works were devoted to the study of the impact of OMWW on soil microbiological activity such as respiration and bacterial and fungal populations (Chatzistathis and Koutsos 2017; Chehab et al. 2019; Meftah et al. 2019). The presence of high concentrations of certain OMWW constituents such as polyphenols may induce to an antimicrobial effect on some microbial groups and phytotoxic effect of the soil (Barbera et al. 2013; Ayed et al. 2005). Polyphenols can also have phytotoxic effect towards plants (Ahmali et al. 2020). On the other hand, the increasing of pH, EC and enrichment of soil by carbon and some nutrients such as nitrogen, phosphorus and potassium after OMWW spreading could increase the concentration of microbial groups such as fungi, yeast and actinomycetes (Chehab et al. 2019; El Hassani et al. 2020). As a wastewater, OMWW must be treated before being discharged. Different treatment methods have been studied in lab and in large scale including physical (dilution, filtration, evaporation, sedimentation and centrifugation), biological (activated sludge) and physicochemical (flocculation, precipitation, adsorption, chemical oxidation, ion exchange and coagulation) treatments. However, these treatments were either not able to reduce organic loads and toxicity to acceptable limits or are relatively expensive as energy or large quantities of chemicals are required (Pelendridou et al. 2014; Paraskeva and Diamadopoulos 2006; Mantzavinos and Kalogerakis 2005; Galanakis 2017). Different biological treatments were also studied such as microorganism treatment, aerobic and anaerobic bioreactor, composting and CWs (Achak et al. 2009, 2019; Ouzounidou et al. 2010; Muktadirul Bari Chowdhury et al. 2013; Paraskeva and Diamadopoulos 2006; Mantzavinos and Kalogerakis 2005). Thanks to its low construction and operation costs, the environmental benefits and the involvement of biological, chemical and physical phenomena, the CW was recently applied for the treatment of diverse types of wastewaters, including the most polluted ones such as industrial tannery wastewater (Tiglyene et al. 2005; Calheiros et al. 2007; Saeed et al. 2012), pulp and paper industry wastewater (Knight et al. 2000), acid mine drainage wastewater (Kleinmann and Girts 1987), swine wastewater (Li et al. 2020), industrial dairy wastewater (Yazdani and Golestani 2019), wine wastewater (Laura et al. 2021) and OMWW (Herouvim et al. 2011; Michailides et al. 2015; Kapellakis et al. 2012; Tatoulis et al. 2017; Achak et al. 2019; Del Bubba et al. 2004; El Ghadraoui et al. 2020). In CW, the major part of the treatment occurs in the substrate, also known as media, support matrix/material or filling material (Wang et al. 2018). Conventional substrates, such as sand, gravel and soil, are mainly used in order to support the plants in CWs with marginal function on nutrient (especially phosphorus) and some specific pollutant removal (Wang et al. 2018; Zhu et al. 2011). In the recent years, novel materials such as pozzolan (El Ghadraoui et al. 2020), tire chips (Chyan et al. 2013), oyster shells (Park and Polprasert 2008) and construction wastes (Shi et al. 2017) have proven their efficiency in the increase of the treatment capacity and the prevention from clogging issues in constructed wetlands.

To the best of our knowledge, the current work is the first documenting the behaviour of both microbiological and physicochemical characteristics of vertical flow CW substrate after treating a mixture of urban wastewater (UW) and olive mill wastewaters (OMWW). Based on the aforementioned considerations, the aim of this work is to evaluate the behaviour of microbiological and physicochemical proprieties of vertical flow constructed wetland with pozzolan layer treating a mixture of UW and OMWW.

Material and methods

Experimental site

The treatment study was conducted for a period of over 1 year in the botanic garden inside the Faculty of Science Semlalia-Marrakech (Morocco). In this area, two pilot vertical flow CWs were established (Fig. 1).

The pilots were built from a polyvinyl chloride circular tank (height 0.60 m and surface area 0.24 m^2) and filled from the top with 30 cm of sand (0.25/0.40 mm) as infiltration layer followed by 10 cm of pozzolan (5/20 mm) as transition layer and 10 cm of gravel (20/40 mm) as drainage layer. The full details are reported in the previous work (El Ghadraoui et al. 2020). Both pilots were planted with *Phragmites australis.* In the bottom of the tanks, a drain was installed in order to collect the water after its treatment. Both influents and effluents were monitored for a period of over 1 year in order to highlight the efficiency of the two CWs. The first pilot (Fig. 1a) was feed with a mixture of OMWW and UW (Mixture) with the following proportions, 89.9% organic load of OMWW and 10.1% of UW (El Ghadraoui et al. 2020). The second pilot (Fig. 1b) was fed with only UW. Both pilots were fed using peristaltic pump according to the following program: 1-day alimentation and 2-day rest respecting an organic loading rate of 366 g of COD/m^2/day.

Sampling

Liquid sampling

OMWW used in this study was collected from an extraction unit of olive oil working with a traditional extraction system

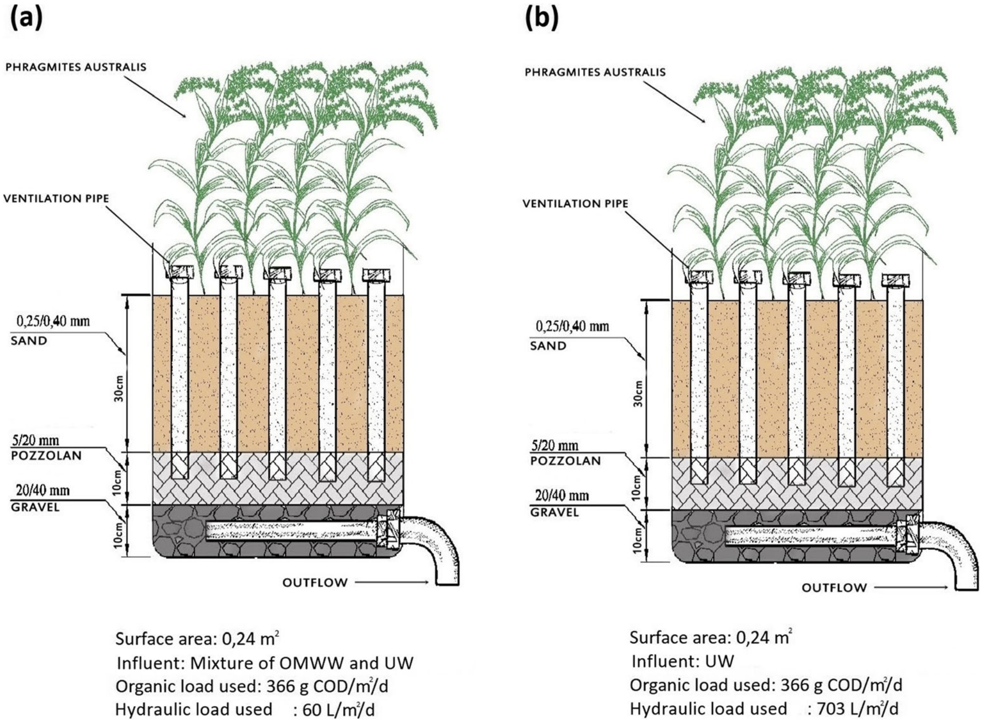


Fig. 1 Diagram of the pilot scale-constructed wetlands

(press) located in Rass El Ain 50 km on the N8 of Marrakech City (Morocco). The sampling was achieved during the month of February 2016. The UW used to perform the mixture is collected from the inlet of Marrakech wastewater treatment plant (activated sludge) each week in order to preserve the biomass present in that wastewater.

Solid sampling

One sampling campaign was conducted after 1 year of the system functioning. Three samples of sand from the CW treating the mixture (S-Mixture) and sand from the CW treating UW (S-UW) were collected using a soil sampler probe at 3 different depths (0–10 cm, 10–20 cm and 20–30cm) and compared to raw sand (RS) in order to evaluate the effect of the mixture and UW on both physicochemical and microbiological parameters inside the CWs.

Influent and effluent analysis

EC, pH and total dissolved salts (TDS) were measured at room temperature with a multi-probe parameter type (HANNA HI 9829, Romania). Total suspended solids (TSS) were measured following (T90-105, AFNOR 1997) using a filtration membrane (0.45μm), COD determination is made by the potassium dichromate method according to AFNOR standard (T 90-101, AFNOR 1988). Total phosphorus (TP) was performed following the protocol (T90-023, AFNOR 1982). Ortho-phosphorus (PO_4^{3-}) analysis is performed according to the protocol (T90-022, AFNOR 1982); ammonia (NH_4^+) is determined by a colorimetric technique according to the AFNOR standard (T90-015, AFNOR 2000); nitrites (NO_2^-) are determined after diazotization according to (T90-013, AFNOR 1985); nitrates (NO_3^-) are reduced to nitrites by passage over a copper cadmium column (Rodier et al. 2009). Measurement of sulphate is carried out according to Rodier et al. (2009).

Substrate physicochemical analysis

EC and pH were determined using a multi-parameter instrument (HANNA HI 9829, Romania) by mixing 10 g of extracted sand with 50 ml of distilled water. Total organic carbon (TOC) was determined following the Anne method (Aubert 1978) which consisted on the oxidation of the organic matter carbon by potassium dichromate in sulphuric medium until release of CO_2; the excess of dichromate is drawn by a solution of iron sulphate and ammonium (Mohr salt) in the presence of an indicator diphenylamine. Total nitrogen (TN) was quantified using the method (ISO 11261, AFNOR 1995), and the sample is mineralized in sulphuric acid medium in the presence of copper (II) and a catalyst (titanium oxide). Under the conditions of mineralization, organic nitrogen is recovered in the ammonium form. The ammonium ions are converted to ammonia by passing in an alkaline medium. NH_3 is driven to the water vapour and the condensate collected dose volumetric acid/base titration.

For the analysis of polyphenols at different depths, the Macheix method (Macheix et al. 2018) was adapted to determine phenolic compounds. Substrate sample of 10 g each was shaken in 20 ml cold methanol (80% v/v) for 15 min and the mixture was centrifuged for 3 min at 5000 rpm at 4°C. This step was repeated three times before the supernatants were evaporated to remove methanol. A solution of ammonium sulphate (40% v/v) was added to the extract followed by meta-phosphoric acid solution of 20% (1/10 v/v). This phase was followed by depigmentation and defatting of with petroleum ether (v/2). The extract was purified by ethylene acetate (v/v) and evaporated to dryness at 35°C with a rotary evaporator and the residue was recovered in 2 ml of Grade HPLC pure methanol before being analysed using the KNAUER HPLC model AZURA (Berlin, Germany).

Substrate bacteriological analysis

Microbial counts of CW substrates initially focused on the enumeration of total flora, yeasts and fungi. The microbiological analyses of samples were carried out upon receipt in the laboratory in order to avoid any modification of the initial microbial concentration. After homogenization of sand samples, a series of dilutions in sterile physiological saline is performed (0.9% NaCl). A volume of 0.1 ml of the appropriate dilution is plated on Petri dishes containing the appropriate agar medium at the rate of three repetitions by dilution. Nutrient agar (BK185HA, Beauvais, FR) is used at pH 7 for total flora community, and the incubation of spread boxes is carried out at 37°C for 24 h. Sabouraud dextrose agar (L007492, Maryland, USA) culture medium is used to determine fungi to which 25 μg/ml of Chloramphenicol (IB02080, Dubuque, USA) was added as antibiotics to prevent microbial growth. The incubation of the inoculated dishes is carried out at 30 °C for 3–7 days. For yeast, the culture medium used is Peptone Dextrose Agar (242720, Maryland, USA), and incubation is carried out at 30°C for 48–72 h.

Statistical analysis

Statistical analyses were done using statistical software SPSS Statistics 20. Three repeats were performed in this study. They have been expressed in mean ± standard deviation, using analysis of variance ANOVA (Analysis of Variance). Pearson correlation and t test with P = 0.05 were also used.

Results and discussion

The study was carried out in the spring period characterized by a temperature between 10 and 22°C in March and between 18 and 32°C in June. This period was characterized by no rain (Fig. 2).

Influent and effluent characterization

Table 1 shows the physicochemical proprieties of the studied wastewaters. OMWW is acidic (pH=5.01), characterized by a high conductivity 28.23 mS/cm, a high organic content evaluated in terms of COD 264.05 g/l and high content of total suspended solids of 2066 mg/l.

The concentration of polyphenols is particularly high of 8.73g/l. The characteristics of OMWW are often variable and depend on several factors (e.g. olive variety, extraction method, fruit maturation). However, similar results have been reported by several authors in the literature (Azbar et al. 2004; Aissam 2003; Piotrowska et al. 2011; Aktas et al. 2001).

The concentration of OMWW was decreased by using UW as diluent. The pH went from 5.01 to 7.33, conductivity decreased by 84%, TSS decreased by 72%, COD decreased by 96% and total polyphenols decreased by 98%. The practical results in Table 1 can be slightly different from the theoretical values calculated based on a dilution factor of 9:1 (UW: OMWW). This lack of correlation is basically due to the use of large volume materials (usually less precise) for the preparation of important volumes of the mixture. Since the OMWW is highly concentrated the slightest drop could change drastically the concentration especially for COD. This would not impact the results from the CW pilot as for each campaign both the influent and the effluent were analysed.

The aim of using UW for the dilution of OMWW and not tap water (as it is mostly used in literature) is to simultaneously treat two types of sewage at once and to increase the efficiency of the system by providing a bacterial flora that will optimize the operation of the CW, so through this method we are witnessing a conservation of water and energy resources.

Treatment efficiency

The removal efficiency of the CW pilot towards the mixture is well detailed in the author’s previous work (El Ghadraoui et al. 2020). Both pilot units were monitored for a period of over 1 year in order to demonstrate their performance regarding the treatment of different influents (Mixture and UW). The obtained results (Table 2) show that the CW pilot unit treating the mixture presents a remarkable performance towards different pollutants despite the complexity and the high organic load of the mixture.

The pilot treating the mixture managed to remove 99%, 91%, 89%, 94%, 94%, 58%, 92% and 95% for TSS, COD, total polyphenols, PO_4^{3-}, P, SO_4^{2-}, NO_2^- and NH_4^+ respectively (El Ghadraoui et al. 2020). The pilot treating UW allowed to determine the following removal efficiencies; 99%, 85%, 90%, 91%, 46%, 89%, 87% for TSS, COD, PO_4^{3-}, P, SO_4^{2-}, NO_2^- and NH_4^+ respectively. Similar or less efficient removal performances were reported by the authors regarding the treatment of OMWW+UW mixture by CW. For

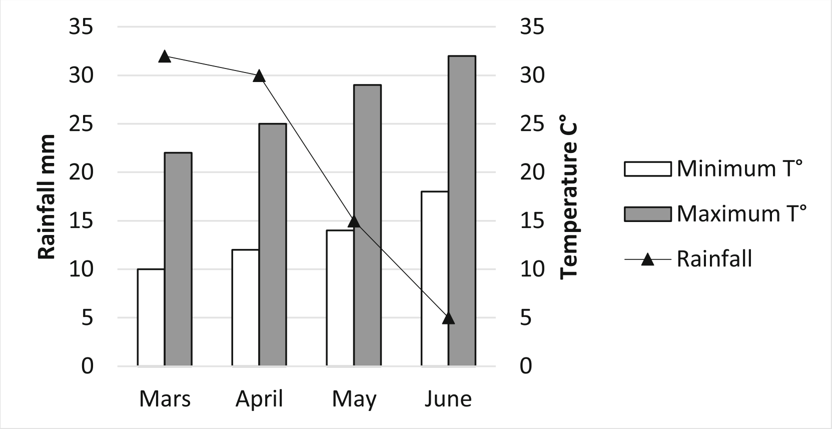


Fig. 2 Climatic diagram representing the rainfall, maximum and minimum air temperature during the experimental period

TSS, a removal efficiency of 70% was reported by Achak et al. (2011); several authors have reported a COD removal reaching 73% (Yalcuk et al. 2010; Herouvim et al. 2011); for polyphenols, the performances reported in literature were around 70% (Kapellakis et al. 2008; Herouvim et al. 2011) and 95% (Achak et al. 2011); for NH_4^+, the removal attained 75% (Achak et al. 2011) and 49% (Yalcuk et al. 2010). An elimination of 95% (Herouvim et al. 2011) and 87% (Yalcuk et al. 2010) were reported regarding the phosphorus concentration.

Substrate physicochemical characteristics

The result of RS, S-Mixture and S-UW physicochemical analyses are shown in Table 3.

RS: Raw sand; S-UW: sand from CW treating UW; S-Mixture sand from CW treating OMWW; EC: electrical conductivity; TDS: total dissolved salts; TOC: total organic carbon; TN: total nitrogen; C/N: carbon/nitrogen ratio

Table 1 Physicochemical characteristics of urban wastewater (UW), crude olive mill wastewater (OMWW) and a mixture of olive mill wastewater and urban wastewater (Mixture) (89.9% and 10.1% organic load, respectively) (mean of 3 replicates ± standard deviation) (El Ghadraoui et al. 2020)

Parameters	UW	OMWW	Mixture
pH	7.07	5.01	7.26
EC, mS/cm	2.21	28.23	4.445
TDS, g/l	0.32	22.10	2.22
COD, g/l	0.51±0.41	264.05±11.49	6.10±0.54
TSS, mg/l	228.33±13.5	2066±11.26	577.78±13.87
Total polyphenols, mg/l	0.01±0.004	8732±0.43	131±3.27
PO_4^{3-}, mg/l	0.82±0.06	31.14±0.65	9.45±0.46
P, mg/l	0.95±0.06	41.61±4.37	10.19±0.48
NH_4+, mg/l	12.95±0.52	6.33±0.30	12.40±0.94
NO_{3-}, mg/l	0.04±0.01	1.32±0.05	0.22±0.04
NO_{2-}, mg/l	1.25±0.08	96.23±9.41	2.04±0.08
SO_4^{2-}, mg/l	136.6±12.58	1320±0.05	232.6±33.99

The pH of RS was neutral (7.27). The monitoring of substrate characteristic behaviour in different depths helps to conclude that the pH remains always neutral in both pilots (Fig. 3-A). This is probably due to the similarity of UW and the mixture pH (see Table 1). These results are similar to those reported by several authors stipulating that the pH remains unchangeable in the sand after the application of OMWW. Piotrowska et al. (2011) reported values of 8.6 and 8.7 for raw substrate and for the substrate after OMWW application respectively after 42 days of study. Meftah et al. (2019) reported values of 7.56 and 7.36 for raw substrate and for the substrate after OMWW application respectively. The authors have also demonstrated that in the case of a highly acidic or high applied load of OMWW, a slight decrease of pH is noticed. However, this decrease occurred only in short term as after few weeks, the pH return to neutral (Piotrowska et al. 2011; Meftah et al. 2019).

Table 3 and Fig. 3-B show that the treatment of the mixture by the CW have resulted in an increase of EC in the first two layers (0–10 and 10–20 cm), as EC increased from 0.134 to

Table 2 Removal efficiency achieved by the investigated pilots (El Ghadraoui et al. 2020)

Parameters	Pilots	
	Mixture pilot (%)	UW pilot (%)
TSS	99	99
COD	91	85
Total polyphenols	89	–
$PO_4{}^{3}{}_{-}$	94	90
P	94	91
$SO_4{}^{2}{}_{-}$	58	46
NO_{2-}	92	89
NH_4+	95	87

Table 3 Substrate physicochemical characteristics per kg of dry sand (mean of 3 replicates ± standard deviation)

Parameters	Unit	RS	S-UW	S-Mixture
Layer 0–10cm				
pH		7.27±0.14	7.24±0.21	7.28±0.14
EC	mS/cm	0.13±0.01	0.18±0.01	0.22±0.01
TDS	mg/kg	65.45±1.3	89.36±2.7	108.67±4.34
Total polyphenols	mg/kg	0.06±0.01	0.9±0.05	2.1±0.23
TN	mg/kg	0.11±0.01	0.14±0.01	0.45±0.06
TOC	%	0.64±0.07	0.97±0.11	6.84±0.75
C/N		5.81±0.7	6.92±0.83	15.2±1.82
Layer 10–20cm				
pH		7.24±0.14	7.27±0.21	7.31±0.14
EC	mS/cm	0.13±0.01	0.23±0.01	0.28±0.01
TDS	mg/kg	64.33±1.3	100.67 ±3.02	135.3±5.41
Total polyphenols	mg/kg	0.05±0.01	0.8±0.04	1.9±0.2
TN	mg/kg	0.09±0.01	0.13±0.01	0.43±0.06
TOC	%	0.63±0.07	0.88±0.1	6.41±0.7
C/N		5.72±0.68	6.76±0.81	14.9±1.8
Layer 20–30cm				
pH		7.22±0.14	7.27±0.21	7.35±0.14
EC	mS/cm	0.13±0.01	0.17±0.01	0.21±0.01
TDS	mg/kg	64.29±1.3	79.33±2.37	93.67±3.74
Total polyphenols	mg/kg	0.03±0.01	0.7±0.04	1.6±0.2
TN	mg/kg	0.06±0.01	0.12±0.01	0.41±0.06
TOC	%	0.59±0.07	0.8±0.096	5.88±0.64
C/N		5.36±0.64	6.66±0.8	14.34±1.72

0.222 mS/cm in 0–10 cm and from 0.131 to 0.283 mS/cm in 10–20 cm. TDS mean concentrations were also increased in the first two layers as the concentration went from 65.45 to 108.67 mg/kg in 0–10cm and from 64.33 to 135.3 mg/kg in 10–20cm (Fig. 3-C). The same results were reported by several authors (Di Serio et al. 2008; Magdich et al. 2016; Barbera et al. 2013; Karpouzas et al. 2010) demonstrating that EC and TDS in the substrate in which OMWW were applied increases in the first 20cm. This increase in EC and TDS is undoubtedly the results of the physical trapping of salts in sand layers (Corwin and Yemoto 2020). However, it is observed that the conductivity and TDS is slightly lower in the 20–30 cm layer, since EC decreased from 0.283 to 0.216 mS/cm and TDS from 135.3 to 93.67 mg/kg. The same results were reported by Meftah et al. (2019) demonstrating that the EC and TDS decrease starting from 20 cm. The observed increase in conductivity and in total dissolved salts remains temporary over time (Chiesura et al. 2005).

The results in Table 3 and Fig. 3-E demonstrate also the behaviour of TOC inside the different pilots. It shows that raw sand is very poor in total organic carbon ≈0.64%. It was also observed that after the treatment of the mixture in the CW, the concentration of TOC has increased significantly as the value jumped from 0.86 to 6.84% in the first layer (0–10cm). This rise is undoubtedly due to the high organic load applied on the pilot (366 g COD/m^2/day). The same results were reported by Piotrowska et al. (2011), Di Serio et al. (2008). Figure 3-E shows that the highest concentration of organic matter is located in the first 0–10 cm layer. This is possibly due to the physical barrier role played by the fine sand which captures the particulate organic matter. Figure 3-E also shows that the concentration of TOC slightly decreases with depth as it went from 6.84 to 6.41% and 5.88% respectively in the 10–20-cm and 20–30-cm layers.

According to the results presented in Table 3 and Fig. 3-D, the concentration of total nitrogen has significantly increased after the treatment of the mixture. The measured concentrations in S-Mixture were 0.45, 0.43 and 0.41 mg/kg of dry sand whereas for RS, the concentrations were 0.11, 0.9 and 0.6 mg/kg of dry sand respectively for 0–10, 10–20 and 20–30-cm depths. Similar results were reported in the literature as authors have demonstrated that the concentration of nitrogen in the substrate increases when the latter is in contact with OMWW (Piotrowska et al. 2011; Meftah et al. 2019). This significant increase is probably due to the high concentration of nitrogen in the mixture as shown in Table 3.

Data demonstrated in Fig. 3-F show that C/N ratio has been increased by a factor of three after the treatment of the mixture

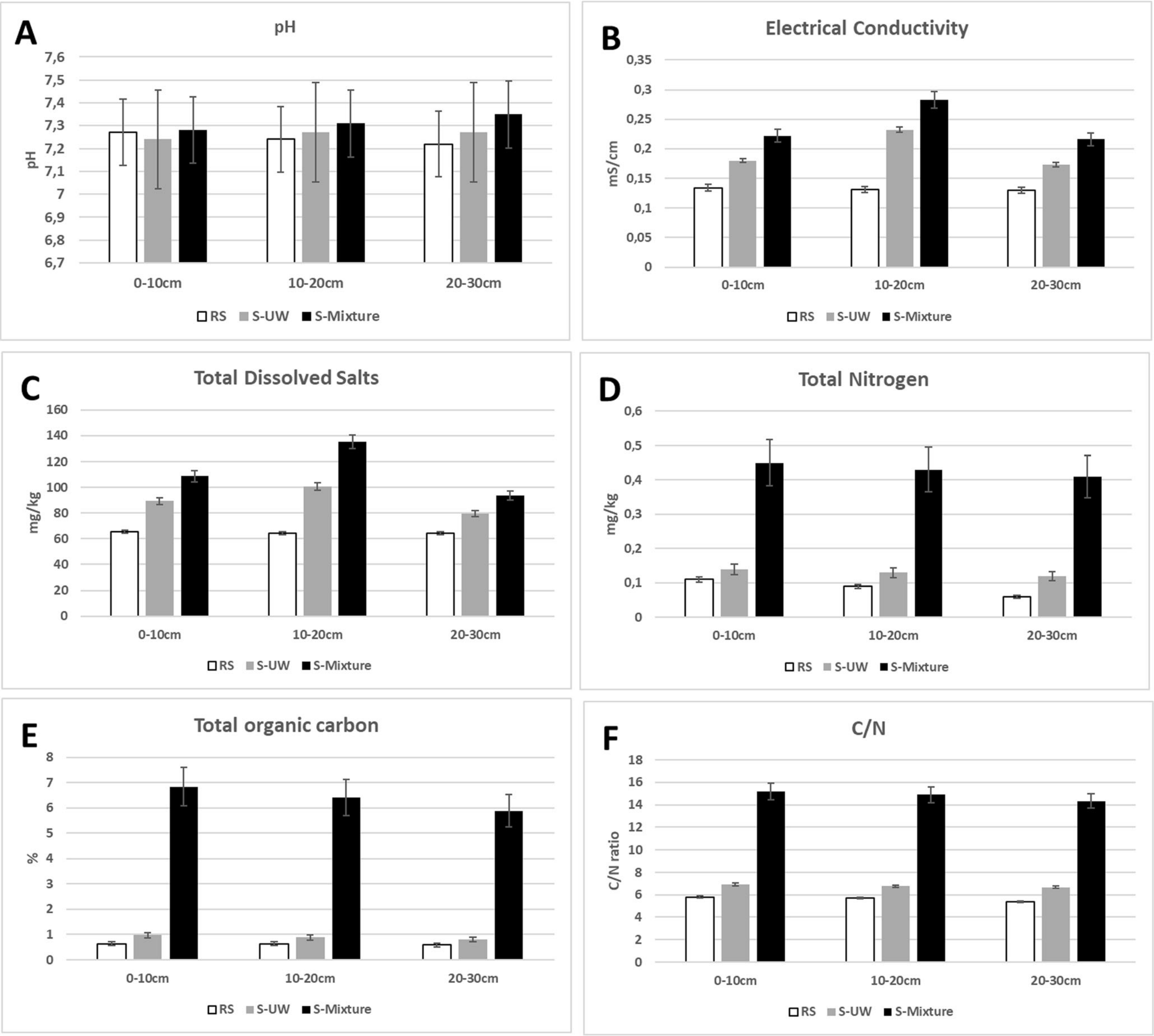


Fig. 3 Physicochemical characteristics of raw sand (RS), sand from CW receiving urban wastewater (S-UW) and sand from CW receiving the mixture (S-Mixture). **A**-pH, **B**-Electrical conductivity, **C**-total dissolved salts, **D**-Total nitrogen, **E**-total organic carbon, **F**-C/N ratio

by the CW, since the value of the C/N ratio in the RS was 5.81 whereas for S-Mixture, C/N ratio in the first 0–10-cm layer is 15.2. These results are supported by other studies, as they report that the application of OMWW on a substrate may result in the increase of organic carbon and therefore the increase of the C/N ratio (Paredes et al. 1987; Di Serio et al. 2008; Piotrowska et al. 2011; Barbera et al. 2013). In this study, the highest C/N ratio was observed in the first layer (0–10 cm). The results in Fig. 3-F show that the C/N ratio tends to decrease with the increase of depth (Meftah et al. 2019).

For polyphenols, the data show that the concentrations in RS are very low of 0.06, 0.05 and 0.03 g/kg of dry sand respectively for 0–10, 10–20 and 20–30 cm (Table 3 and Fig. 4).

In S-UW, the concentrations of polyphenols are slightly higher than those observed in RS since the values were 0.9, 0.8 and 0.7 g/kg of dry sand respectively for 0–10, 10–20 and 20–30 cm. In S-Mixture, the concentrations of polyphenols were significantly higher compared to RS and S-UW as the concentrations were 2.1, 1.9 and 1.6 g/kg of dry sand respectively for 0–10, 10–20 and 20–30 cm (Table 3). The results show also that the concentration of polyphenols decreases with the increase of depth. Similar results were reported by Di Serio et al. (2008) as the authors demonstrated that the concentration of total polyphenols in the substrate increase after the contact with the OMWW. In the same study, the

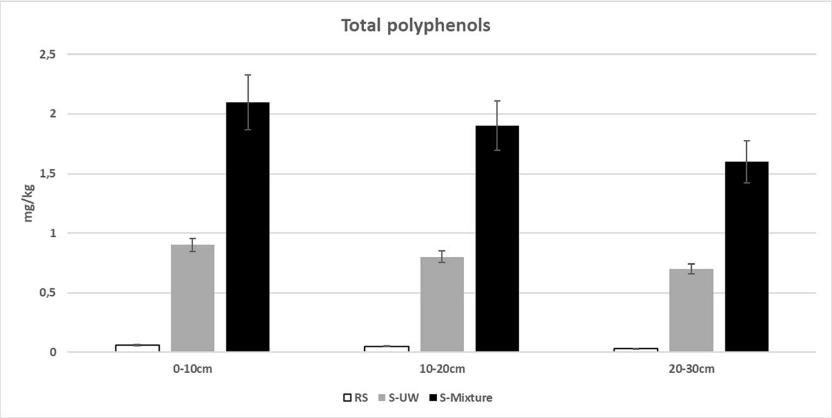


Fig. 4 Concentration of total polyphenols in different substrates

authors have shown that the concentration of polyphenols decreases by the increase of depth. In another work, the author has demonstrated that when OMWW is applied on a substrate, the polyphenol concentration increases and the majority of phenolic compounds are located in the upper layer (Mekki et al. 2007). The same authors have shown that the concentration of polyphenols decreases quickly from 0 to 25 cm then continued to decrease slightly with depth but remained detectable at 120 cm.

Substrate microbiological proprieties

Microbial counts were determined in order to compare the total flora, fungi and yeast in the two CW substrates (S-UW and S-Mixture) receiving respectively UW and the UW/OMWW mixture. Figure 5 shows that generally speaking for S-UW, a dominance of total flora was observed with a mean concentration of 9.68E+06 CFU/g of dry sand followed by fungi and yeast with a respective mean concentration of 1.29E+04 and 2.49E+04 CFU/g of dry sand.

The same results were found for S-Mixture where total flora was the most dominant group followed by yeast and fungi respectively with the following means concentrations 1.02E+07, 5.87E+06 and 2.96E+05 CFU/g of dry sand. The application of T test on microbial diversity for both pilot systems revealed no significant difference between the abundance of total flora in S-UW and S-Mixture (P = 0.7) and fungi (P = 0.54) whereas the opposite for yeast where the difference is very significant (P = 0.01). The increasing of microbial community was reported by El Hassani et al. (2020) who demonstrated that the abundance of soil total microflora is enhanced after OMWW application. This change in the microbial community was explained by several authors as it could result from the interactions between different factors such as micro environmental changes (lowered oxidative conditions, strong competition for mineral nitrogen and the availability of phenolic compounds) and the selective inhibition of other microbial groups by phenols and altered C-sources (Karpouzas et al. 2010). It has been suggested that the spreading of OMWW impacted the structure of the soil microbial communities by affecting the nutritional status of the soil (Rousidou et al. 2010). The same authors related the changes of microbial community to the modification of soil structure that occurs after the application of OMWW on the substrate.

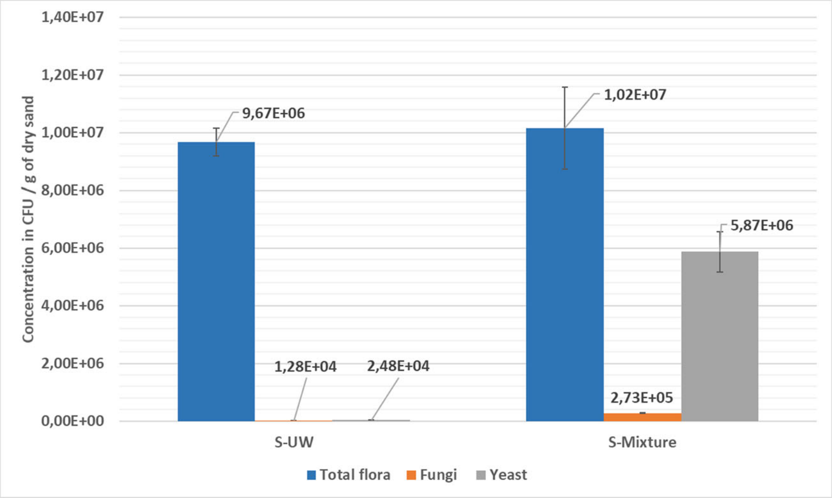


Fig. 5 Microbiological characteristics of S-UW and S-Mixture

The abundance of actinomycetes, Gram-positive bacteria, fungi and arbuscular mycorrhizal fungi are related to variations with soil total carbon and total nitrogen (Zhao et al. 2019). Therefore, the increasing of total flora community after treatment of the mixture by the CW has been justified as the physicochemical analysis on S-Mixture have shown an increasing of pH, EC, TDS, TN, TOC and C/N ratio. In this study, the increasing of fungi and yeast can justify the high performance of the studied CW regarding the treatment of the mixture (see Table 2) (El Ghadraoui et al. 2020). As it is reported in the literature, these microbial groups are mainly responsible for the degradation of organic fraction especially phenolic compounds (Mutabaruka et al. 2007; Sinsabaugh 2010; Di Serio et al. 2008).

The observed increase of microbial community and especially for yeast can be correlated with removal efficiency as it is reported in the literature that microbial communities such as yeast and fungi are responsible for the removal of organic matter especially the non-easily degradable such as polyphenols. Morillo et al. (2008) studied the bioremediation of OMWW and stated that the increase in the relative fungal/bacterial ratio was accompanied by high polyphenolic and organic matter reduction. It is also reported in the literature that this microbial groups are mainly responsible for the degradation of organic fraction especially phenolic compounds (Mutabaruka et al. 2007; Sinsabaugh 2010; Di Serio et al. 2008).

Amaral et al. (2010) reported that several microbial strains were tested for their ability to remove organic matter from diluted and undiluted OMWW. The most used were strains of *Geotrichum candidum*, but other yeast species such as *Candida tropicalis*, *C. rugosa*, *C. cylindracea*, *Trichosporon cutaneum* and *Yarrowia lipolytica* have been already reported as able to remove some organic matter from effluents.

Table 4 Microbiological characteristics of CW pilot plant substrate receiving urban wastewater (S-UW) and CW pilot plant receiving the mixture (S-Mixture) at different depths (mean of 3 replicates ± standard deviation)

Parameters	Unit	S-UW	S-Mixture
		Layer 0–10cm	
Total flora	CFU/g	$(1.39 \pm 0.06) \times 10^7$	$(1.02 \pm 0.07) \times 10^7$
Fungi		$(2.14 \pm 0.1) \times 10^4$	$(3.4 \pm 0.1) \times 10^5$
Yeast		$(3.1 \pm 0.06) \times 10^4$	$(3.43 \pm 0.1) \times 10^6$
		Layer 10–20cm	
Total flora	CFU/g	$(9.20 \pm 0.46) \times 10^6$	$(1.35 \pm 0.09) \times 10^7$
Fungi		$(1.45 \pm 0.07) \times 10^4$	$(4.69 \pm 0.14) \times 10^5$
Yeast		$(4.33 \pm 0.08) \times 10^4$	$(1.37 \pm 0.04) \times 10^7$
		Layer 20–30cm	
Total flora	CFU/g	$(5.90 \pm 0.29) \times 10^6$	$(6.78 \pm 0.47) \times 10^6$
Fungi		$(2.60 \pm 0.13) \times 10^3$	$(7.5 \pm 0.02) \times 10^4$
Yeast		$(1.98 \pm 0.03) \times 10^2$	$(4.82 \pm 0.24) \times 10^5$

ANOVA test applied to highlight the variation of microbial counts in both investigated pilots as a function of depth showed no significant difference for total flora and fungi. However, yeast has shown a significant difference ($P=0.01$) in the S-Mixture CW.

RS: Raw sand; S-UW: sand from CW treating UW; S-Mixture sand from CW treating OMWW

Table 4 and Fig. 6-A show that in the CW receiving UW a slight difference was noticed for total flora count as concentrations were 1.39×10^7, 9.2×10^6 and 5.9×10^6 CFU/g of dry sand respectively for layers 0–10, 10–20 and 20–30cm. In terms of fungi and yeast, no significant difference was observed. Fungi's concentrations were 2.14×10^4, 1.45×10^4 and 2.6×10^3 CFU/g of dry sand respectively for 0–10, 10–20 and 20–30-cm layers. Yeast concentrations in the same layers were respectively 3.1×10^4, 4.33×10^4 and 1.98×10^2 CFU/g of dry sand.

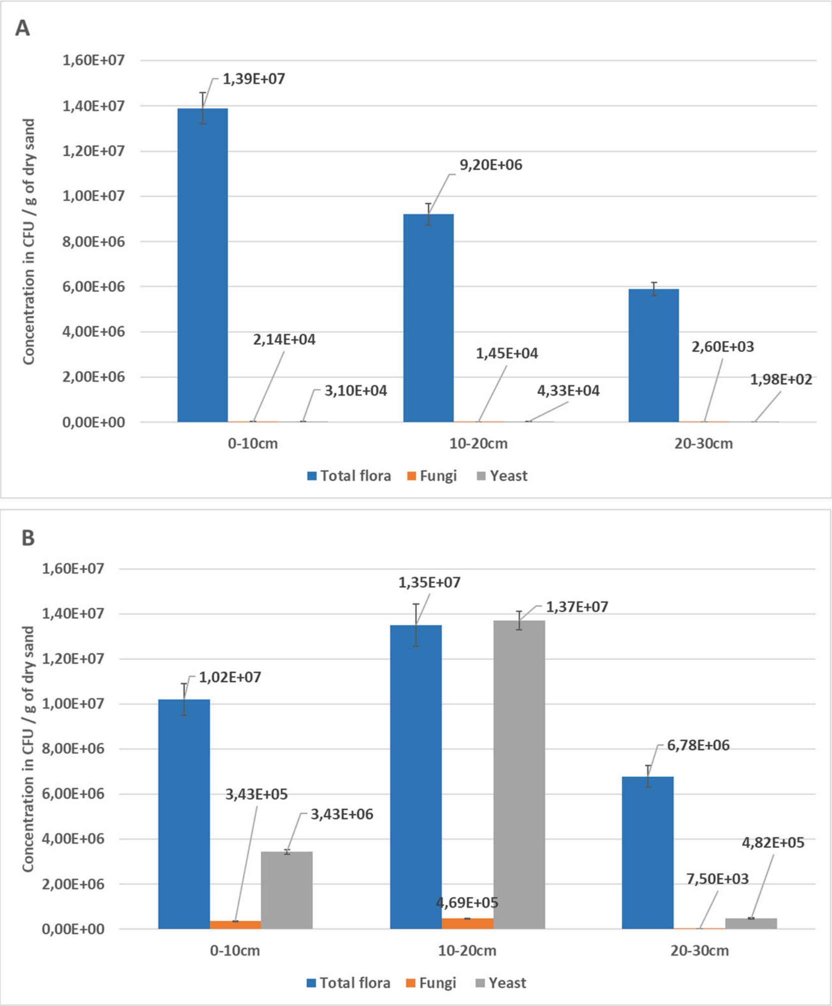
Figure 6-B shows that in CW treating the mixture, the highest concentrations of total flora, fungi and yeast are located in the first 20 cm with a massive increase (×10) in the content of yeast as it increased from 3.43×10^6 to 1.37×10^7 CFU/g of dry sand. This increase can be explained as high concentrations of yeast can be imported by OMWW since yeast are the dominant microorganisms in OMWW when compared to other groups of microorganisms (Amaral et al. 2010; Grafias et al. 2010). Moreover, compared to other microbial groups, yeast have a much higher growth rate and they can resist to the toxicity of phenolic compounds (Mendonça et al. 2004).

In the literature, the high concentration of microbial groups in the first 20 cm was confirmed by the authors demonstrating that diversity index was highest in the top layer (0–10 cm), and that the relative abundances of bacteria and fungi generally decreased significantly at 0–40 cm depths (Li et al. 2017; Yao et al. 2018). This increase is usually related to the abundant presence of nutritive substances (Nitrogen, Carbon) indispensable for microorganism growth in the surface. However, since the results in the current study demonstrate that the concentration of nutritive substances in all three layers is more or less similar (Fig. 3), we tend to believe that this high abundance could be associated to the physical barrier played by fine sand particles which can block the microorganism in the first layers. Hence, in the first 20 cm the direct addition by the OMWW and to the fast growth process of yeast can explain the high values observed in the first 20 cm layer.

Conclusion

The current work studied the behaviours of physicochemical and microbiological characteristics of a vertical flow

Fig. 6 Evolution of total flora, fungi and yeast in (**A**) S-UW and (**B**) S-Mixture at different depths



constructed wetland substrate after treating a mixture of urban and olive mill wastewaters.

The effects of OMWW application on substrate physicochemical parameters were observed at all depths. Compared to the raw substrate and the substrate from conventional CW, higher concentrations of EC, TDS, TOC, TN, C/N and total polyphenols were detected in the substrate of the CW treating the OMWW. These concentrations were more or less similar at all depths. For microbiological parameters, a general increase of total flora, fungi and yeasts was observed in CW treating the OMWW at all depths in respect to the concentrations detected in the conventional CW substrate. A significant increase in the yeast community was observed in the CW treating the OMWW at a depth of 10–20cm.

From the data collected in this study, we can conclude that changes can occur in the physicochemical and microbiological parameters of the substrate when treating OMWW in CW. However, these changes cause no damage to the CW degradation mechanisms as an increase in the removal efficiency was observed in respect to the conventional CW whilst working under the same conditions.

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Author contribution Conceptualization, methodology, validation, formal analysis, writing the original draft: **Ayoub EL GHADRAOUI and Chaima SAF;** Project administration, formal analysis, writing the original draft: **Naaila OUAZZANI**. Investigation, visualization: **Abdelaali AHMALI, Abdessamed HEJJAJ and Faissal AZIZ;** Conceptualization, writing - review and editing: **Massimo DEL BUBBA;** Resources, supervision, writing - review and editing: **Laila MANDI**. All authors read and approved the final manuscript.

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Declarations

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