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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Research Article

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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 tree depth levels (0-10cm, 10-20cm and 20-30cm) inside a pilot scale CW treating the mixture. In order to compare the obtained results treating the mixture with the conventional case, a control (CW pilot plant treating only UW) was implemented. Result shows that an increase in electrical conductivity (from 134.78 to 222.33µS/cm in 0-10cm and from 131.25 to 283.33 µS/cm in 10-20cm), 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 0-10cm, 10-20cm and 20-30cm respectively), and C/N ratio occurred in the substrate after the treatment of the mixture. As for the microbiological parameters, treating the mixture by CW results in the increasing of yeast and fungi concentration in the substrate which contributes probably to optimize the biodegradation of non-easily degraded organic compound such as polyphenol.

1. Introduction

Olive oil production is an important agro-industrial activity especially in Mediterranean countries (Karpouzas et al. 2010). The most important olive oil-producing countries are Spain, Italy, Greece, and Turkey, followed by Tunisia, Portugal, Morocco, and Algeria (Paraskeva and Diamadopoulos, 2006). World-wide 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-product are produced such as olive pomace and OMWW. OMWW is produced in considerable volumes during the process of olive oil extraction: over 30 million m^3 per year (Niaounakis and Halvadakis 2006). 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 (Azbar et al. 2004; Fountoulakis et al. 2002; Sabbah et al. 2004). 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 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 (Saadi et al. 2007). Many studies were devoted to the study of the impact of OMWW on the biological activity such as respiration, biomass, and hydrolytic activity, bacterial and fungal populations in soil (Alianiello et al. 1998; Saviozzi et al. 2001; Gamba et al. 2005; Paredes et al. 1986; Tardioli et al. 1997). The presence of high concentration of some

OMWW constituents such as polyphenols may induce to an antimicrobial effect on some microbial groups and phytotoxic effect of soil (Barbera et al. 2013; Ayed et al. 2005). On the other hand, the increasing of pH, EC and enrichment of soil by carbon and some nutriment such as nitrogen, phosphorus and potassium after OMWW spreading could increase the concentration of some microbial groups such as fungi, yeast and actinomycetes (Tamoh 1992; Mouncif 1993; Fadil 2002; El Hassani et al. 2005). As a wastewater, OMWW must be treated before being discharged. Different treatment methods have been studied in lab scale and in large scale i.e. physical treatment (dilution, filtration, evaporation, sedimentation, and centrifugation), physicochemical treatment (flocculation, precipitation, adsorption, chemical oxidation, ion exchange and coagulation). However, these treatments were either not able to reduce organic loads and toxicity to acceptable limits or relatively expensive as energy and 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 CW (Achak et al. 2009, 2019; Ouzounidou et al. 2010; 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 (Yazdania and Golestanib 2019), wine wastewater (Flores et al. 2021) and OMWW (Yalcuk et al. 2010; Grafias et al. 2010; Herouvim et al. 2011; Michailides et al. 2015; Kapellakis et al. 2012; Achak et al. 2019; 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 (Yang 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. 2010, Zhu et al. 2011). In the recent years, novel material such as pozzolan (El Ghadraoui et al. 2020), tire chips (Chyan et al. 2013), oyster shell (Park and Polprasert 2008), 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, there is currently no published and the current work is the first documenting the behaviours of microbiological and physicochemical characteristics of vertical flow CW substrate after treating a mixture of urban and OMWW. Based on the aforementioned considerations, the aim of this work is to evaluate the behaviours of microbiological and physicochemical proprieties of vertical flow constructed wetland with pozzolan layer treating a mixture of urban and OMWW.

2. Materials And Methods

2.1 Experimental site

The experience was conducted for a period of over one 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 PVC circular tank and contained 3 layers of substrate, the first from the top is the infiltration layer (30 cm of sand), the second one is the transition layer (10 cm of pozzolan), and the last one is the draining layer (10cm of gravel). 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. The second pilot (Fig. 1b) was feed with only UW. Both pilots were fed using peristaltic pump according to the following program: 1-day alimentation and 2 days rest respecting an organic loading rate of 366 g of COD/m^2/d.

2.2 Sampling

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

2.3 Influent and Effluent analysis

EC, dissolved oxygen and pH, were measured at room temperature with a multi-probe parameters type (HANNA HI 9829, Romania). Total suspended solids (TSS) were measured following (AFNOR T90-105 1983) using a filtration membrane (0,45μm), COD determination is made by the potassium dichromate method according to AFNOR standard (T 90-101, 1983). Total phosphorus (TP) was performed following the protocol (AFNOR T90-023 1983). Ortho-phosphorus (PO_4^{3-}) analysis is performed according to the protocol (AFNOR T90-022 1983), Ammonia (NH_4^+) is determined by a colorimetric technique according to the AFNOR standard (T90-015 1983), Nitrites (NO_2^-) are determined after diazotization according to (AFNOR T90-013 1983), Nitrates (NO_3^-) are reduced to nitrites by passage over a copper cadmium column (RODIER, 2009). Measurement of sulphate is carried out according to (RODIER, 2009).

2.4 Substrate physicochemical analysis

Substrate from the CW treating the mixture (S-Mixture) and substrate from the CW treating UW (S-UW) were analysed at 3 different depths (0-10 cm, 10-20 cm and 20-30cm) and compared to raw substrate (RS) in order to determine physicochemical parameters and their variation in function to the depth. EC and pH were determined using a multi-parameter (HANNA HI 9829, Romania) by mixing 10 g of soil 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 sulfuric 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 (NF ISO 11261), the sample is mineralized in sulfuric 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 vapor and the condensate collected dose volumetric acid / base titration.

For the analysis of polyphenols in different depths, the Macheix method (Macheix et al. 2018) was adapted to determine phenolic compounds. 10 g of each substrate sample was shaken in 20 ml cold methanol (80% v/v) during 15 minutes and the mixture was centrifuged for 3 minutes 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 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 with HPLC.

2.5 Substrate bacteriological analysis

Four sampling campaigns were conducted in order to evaluate the effect of the mixture and UW on the abundance of selected microorganisms inside the CWs. For each pilot plant, 3 soil samples were taken at three different depths: 0-10 cm, 10-20 cm and 20-30 cm using a soil sampler. 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 soil 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, the incubation of spread boxes is carried out at 37°C for 24 hours. Sabouraud dextrose agar (L007492, Maryland, US) culture medium is used to determine fungi in which25 µg/ml of Chloramphenicol (IB02080, Dubuque, US) is added as antibiotics to inhibit any bacterial growth. The incubation of the inoculated dishes is carried out at 30 °C for 3 to 7 days. For yeast the culture medium used is Peptone Dextrose Agar (242720, Maryland, US), incubation is carried out at 30°C for 48 to 72 hours.

2.6 Statistical analysis

Statistical analyses were done using statistical software SPSS Statistics 20. Three repeats were performed in this experience. 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.

3. Results And Discussion

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

3.1 Influents and Effluents characterization

Table 1 shows the physicochemical proprieties of the studied wastewaters. OMWW appear to be acidic 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 2066 mg/l.

Table 1: Physicochemical characteristics of urban wastewater (UW), crude olive mill wastewater (OMWW), mixture of olive mill wastewater and urban wastewater (Mixture) (mean ± standard deviation).

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	0.519±0.416	264.05±11.498	6.10 ± 0.542
TSS, mg/l	228.33±13.50	2066±11.269	577.78 ± 13.87
Total Polyphenols, mg/l	0.01±0.004	8732±0.434	131 ± 3.27
PO_4^{3}-, mg/l	0.82±0.06	31.14±0.651	9.45 ± 0.46
P, g/l	0.95±0.06	41.61±4.376	10.19 ± 0.48
NH_4+, mg/l	12.95±0.52	6.33±0.306	12.40 ±0.94
NO_3-, mg/l	0.04±0.01	1.32±0.055	0.22 ± 0.047
NO_2-, mg/l	1.251±0.08	96.23±9.416	2.04 ± 0.087
SO_4-, mg/l	136.67±12.58	1320±0.055	232.61 ± 33.99

The concentration of polyphenols is particularly high 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).

Performing a mixture using UW act as dilution and allow decreasing most of the pollutants present in the OMWW. 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 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.

3.2 Treatment efficiency

The removal efficiency of the CW pilot toward 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 influent (Mixture and UW). 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.

Table 2: Removal efficiency achieved by the investigated pilots

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%

The pilot managed to remove 99%, 91%, 89%, 94%, 94%, 58%, 92% and 95% for TSS, COD, Total Polyphenol, PO_4^3-, P, SO_4^2-, NO_2^- and NH_4^+ respectively (El Ghadraoui et al. 2020). The monitoring of the control unit designated for the treatment of UW allowed to determine the system performance that are 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 lower performances were reported by authors regarding the treatment of OMWW+UW mixture by CW. For TSS a removal efficiency of 70% was reported by Achak et al. (2010); several authors have reported a COD removal reaching 73% (Yalcuk et al. 2010; Herouvim et al. 2011); for polyphenol 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 elimination of P.

3.3 Substrate physicochemical characteristics

Result of RS, S-Mixture and S-UW physicochemical analyzes are shown in Table 3.

Table 3: Substrate physicochemical characteristics

Parameters	Unit	RS	S-UW	S-Mixture
Layer 0-10cm				
pH		7.27	7.24	7.28
EC	µs/cm	134.78	180.67	222.33
TDS	mg/kg	65.45	89.36	108.67
Total polyphenol	mg/kg	0.06	0.9	2.1
TN	mg/kg	0.11	0.14	0.45
TOC	%	0.64	0.97	6.84
C/N		5.81	6.92	15.2
Layer 10-20cm				
pH		7.24	7.27	7.31
EC	µs/cm	131.25	232.2	283.33
TDS	mg/kg	64.33	100.67	135.3
Total polyphenol	mg/kg	0.05	0.8	1.9
TN	mg/kg	0.09	0.13	0.43
TOC	%	0.63	0.88	6.41
C/N		5.72	6.76	14.90
Layer 20-30cm				
pH		7.22	7.27	7.35
EC	µs/cm	130.87	173.67	216
TDS	mg/kg	64.29	79.33	93.67
Total polyphenol	mg/kg	0.03	0.7	1.6
TN	mg/kg	0.06	0.12	0.41
TOC	%	0.59	0.80	5.88
C/N		5.36	6.66	14.34

The pH of RS was neutral (7.27). The monitoring of substrate characteristic behavior 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 remain unchangeable in the soil 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 experiment. Meftah et al (2019) reported values of 7.56 and 7.36 for raw substrate and for the substrate after OMWW application respectively. 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. Meftah et al (2019) reported values of 7.56 and 6.93 for raw substrate and for the substrate after OMWW application respectively. However, 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 the mean concentration of EC increased from 134.78 to 222.33 µS/cm in 0-10 cm and from 131.25 to 283.33 µS/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 found by several authors (Di Serio et al., 2008; Magdich et al. 2016; Barbera et al. 2013; Karpouzas et al. 2010) which show that EC and TDS in the substrate in which OMWW were applied increases in the first 20cm. This is undoubtedly due to the trapping of salts contained in the mixture at the first layers of the pilot, which causes an increase in TDS and EC (Corwin et al. 2020). However, it is observed that the conductivity and TDS decrease in the 20-30 cm layer, since EC went from 283.33 to 216 µS/cm and TDS went from 135.3 to 93.67 mg/kg. The same results were reported by Meftah et al (2019) who demonstrate that the EC decrease starting from 20 cm. The observed increase in conductivity and in total dissolved salts remains temporary over time (Chiesura et al. 2005).

Results in table 3 and Fig. 3-E demonstrate also the behaviour of TOC inside the different pilots. It shows that raw substrate is very poor in total organic carbon » 0.86% in the first layer. It was also observed that after the treatment of the mixture by 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/d) the same results were reported by (Piotrowska et al. 2011; Di Serio et al. 2008). In Fig. 3-E it is observed that the highest concentration of organic matter is positioned in the first layer 0-10 cm. This is possibly due to the great physical barrier role played by the first layer (sand) which captures the particulate organic matter. Fig. 3-E also shows that the concentration of TOC slightly decreases with depth as it went from 6.84% to 5.89% and 4.57% respectively in the 10-20cm and 20-30cm 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 soil whereas for RS the concentrations were 0.11, 0.9 and 0.6 mg/ kg of dry soil 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 soil increase 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 show in the table 3.

Collected data demonstrated in the Fig. 3-F show that C/N ratio has been multiplied by three after the treatment of the mixture 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 0-10cm layer is 15.2. Several authors tend to confirm these results, as they report that the application of OMWW on a substrate my results 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 concentration of C/N ratio was observed in the first layer (0-10 cm), Results in the Fig. 3-F show that the C/N ratio tend to decrease with the increase of depth (Meftah et al. 2019).

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

In S-UW the concentrations of polyphenols are higher 0.9, 0.8 and 0.7 g/kg of dry soil respectively for 0-10, 10-20 and 20-30 cm. In the substrate of the CW treating the mixture (S-Mixture), the concentrations of polyphenol were significantly higher than in the other substrate as the concentrations were 2.1, 1.9 and 1.6 g/kg of dry soil respectively for 0-10, 10-20 and 20-30 cm (Table 3). The results show also that the concentration of polyphenol 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 polyphenol in the substrate increase after the contact with the OMWW. In the same study, the authors have shown that the concentration of polyphenol 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 polyphenol decreases quickly from 0 to 25 cm then continued to decrease feebly with depth but remained detectable at 120 cm.

3.4 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 mixture. Fig. 5 shows that for S-UW a dominance of total flora was observed with a mean concentration of 9.68E+06 CFU/g of dry soil followed by fungi and yeast with a respective mean concentration of 1.29E+04 and 2.49E+04 CFU/g of dry soil.

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 soil. The application of T test on microbial diversity of both pilot systems revealed no significant difference between the abundance of total flora in S-UW and S-Mixture ($P = 0.7$) also for 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 (2005) who demonstrated that the abundance of soil total microflora is enhanced after OMWW application. This change in the microbial community was explained by multiple 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. The abundance of actinomycetes, Gram-positive bacteria, fungi, and arbuscular mycorrhizal fungi are related to variations with soil total carbon and total nitrogen (Chang 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 justify the high performance of the studied CW regarding the treatment of the mixture (See Table 2). It is 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 et al. 2010; Di Serio et al. 2008).

The removal of polyphenolic compounds in the substrate is mainly due to the biodegradation by fungi (e.g., Ascomycetes and Basidiomycetes) and bacteria (e.g., Pseudomonas) (Sinsabaugh et al. 2010). It was confirmed that planted substrate which is characterized by high amount of organic matter and high polyphenol concentration could facilitate the development of fungi, main microorganisms responsible of polyphenol degradation (Mutabaruka et al. 2007). The soil total polyphenol content is reduced after spreading OMWW because the polyphenols are broken down by specific bacteria, yeast and fungi (Di Serio et al. 2008). ANOVA test applied on the variation of microbial enumeration in both investigated systems regarding the depth showed no significant difference for total flora and fungi. However, yeast has shown a significant difference (P=0.01) in the CW pilot plant receiving the mixture. Table 4 shows that in the CW receiving UW a slight difference was noticed for total flora count as concentrations were $1.39\text{x}10^7$, $9.2\text{x}10^6$ and $5.9\text{x}10^7$ CFU/g of dry soil respectively for layers 0-10, 10-20 and 20-30cm.

Table 4: Microbiological characteristics of raw substrate (RS), CW pilot plant substrate receiving urban wastewater (S-UW) and CW pilot plant receiving the mixture (S-Mixture) at different depths.

Parameters	**Unit**	**S-UW**	**S-Mixture**
	Layer 0-10cm		
Total flora	CFU/g	$1.39.10^7$	$1.02.\ 10^7$
Fungi		$2.14.\ 10^4$	$3.43.\ 10^5$
Yeast		$3.10.\ 10^4$	$3.43.\ 10^6$
	Layer 10-20cm		
Total flora	CFU/g	$9.20.\ 10^6$	$1.35.\ 10^7$
Fungi		$1.45.\ 10^4$	$4.69.\ 10^5$
Yeast		$4.33.\ 10^4$	$1.37.\ 10^7$
	Layer 20-30cm		
Total flora	CFU/g	$5.90.\ 10^6$	$6.78.\ 10^6$
Fungi		$2.60.\ 10^3$	$7.5.\ 10^4$
Yeast		$1.98.\ 10^2$	$4.82.\ 10^5$

In term of fungi and yeast no significant difference was observed. Fungi’s concentrations were $2.14\text{x}10^4$, $1.45\text{x}10^4$ and $2.6\text{x}10^3$ CFU/g of dry soil respectively for 0-10, 10-20 and 20-30cm layers. Yeast concentrations in the same layers were respectively $3.1\text{x}10^4$, $4.33\text{x}10^4$ and $1.98\text{x}10^2$ CFU/g of dry soil. Fig. 6-A shows that in CW treating the mixture the highest concentration of total flora, fungi and yeast is present in the first 20 cm with respective values of $1.35\ \text{x}10^7$, $4.69\ \text{x}10^5$ and $1.37\ \text{x}10^7$ CFU/g of dry soil. This is probably due to abundant presence of nutritive substances (Nitrogen, Carbon) indispensable for microorganism growth in the surface of the CW which is demonstrated in the Fig. 3. Microorganism abundance and diversity tend to decreases significantly with the increase in substrate depth (After 20 cm). These results were confirmed by Li et al (2017) who demonstrated 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 the 0–40 cm depths. Along with these results, Yao et al (2018) reported the decreasing of fungi dominance with the increasing in substrate depth.

4. Conclusion

The current work studied the behaviours of physicochemical and microbiological characteristics of a vertical flow constructed wetland substrate after treating a mixture of urban and Olive Mill wastewaters.

Results have shown that the treatment of the mixture has strongly influenced the physicochemical parameters as the high concentrations of pollutant in the mixture has induced to an increase of EC, TDS, TOC, TN, C/N and the polyphenol. Thus, treating the mixture has also influenced the microbiological parameters as it was observed that after the treatment the concentration of yeast and fungi has increased whereas the concentration of total microflora has decreased which is probably due to the antibacterial effect of the OMWW. It was also demonstrated that this microflora abundance increases in the upper layers of the CW substrate (First 20cm) before decreasing with the increase of depth. In addition, the treatment of the mixture does not affect negatively the removal mechanisms as the highest removal efficiencies were achieved by the CW treating the mixture. This is probably due to the increase of yeast and fungi in the substrate which optimize the degradation of organic compounds especially those hardy degradable by bacteria.

Declarations

Ethics approval and consent to participate

Not applicable

Consent for publication

Not applicable

Availability of data and materials

All data generated or analysed during this study are included in this published article

Competing interests

The authors declare that they have no competing interests

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Authors' contributions

Conceptualization, Methodology, Validation, Formal analysis, writing original draft: **Ayoub EL GHADRAOUI and Chaima SAF;** Project administration, Formal analysis, writing original draft: **Naaila OUAZZANI**. Investigation, Visualization: **Abdelaali AHMALI, Abdessamed HEJJAJ and Faissal AZIZ;**

Conceptualization, Writing - Review & Editing: **Massimo DEL BUBBA;** Resources, Supervision, Writing - Review & Editing: **Laila MANDI**. All authors read and approved the final manuscript.

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Figures

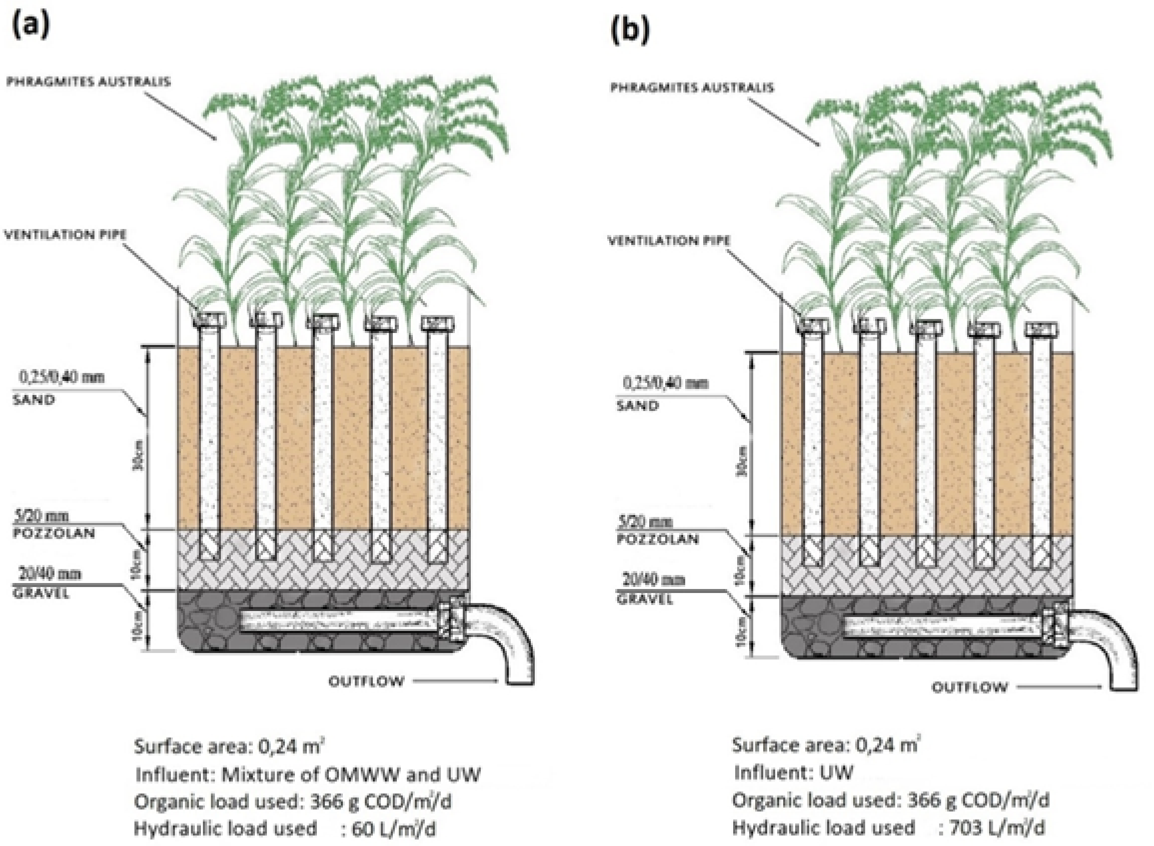

Figure 1

Diagram of the pilot scale constructed wetlands

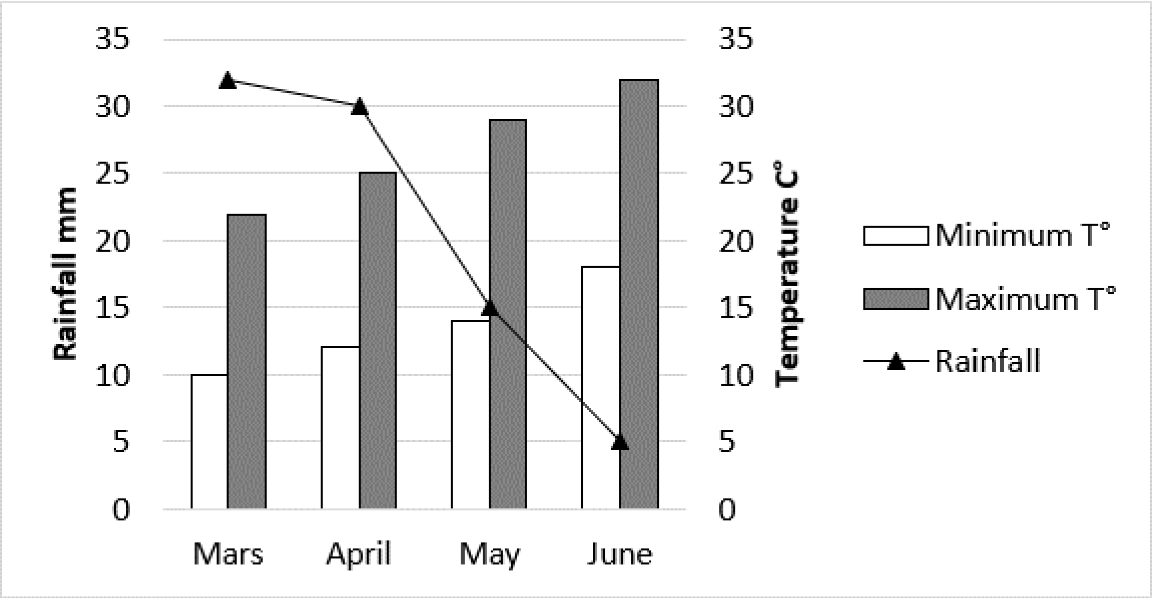

Figure 2

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

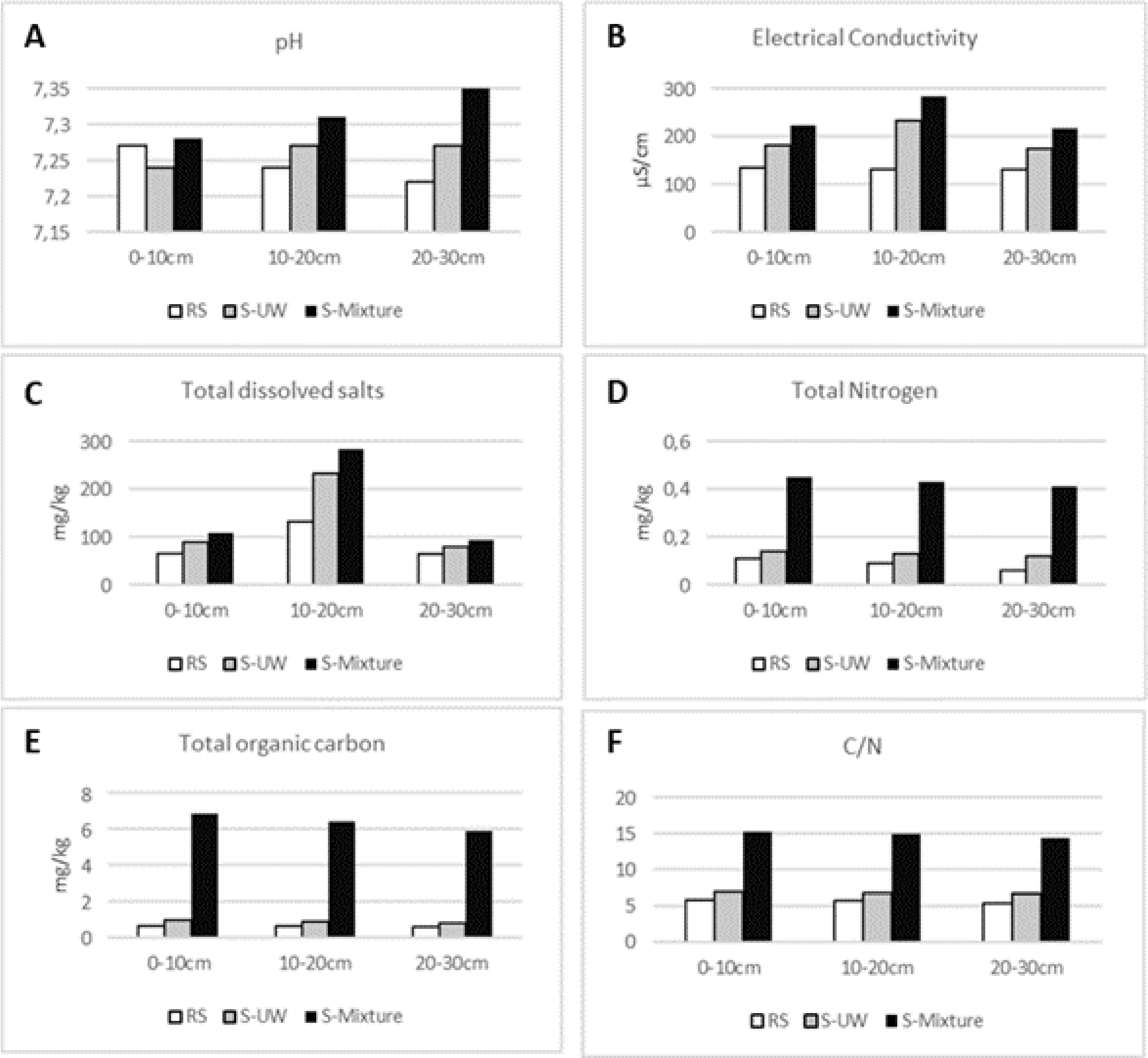

Figure 3

Physicochemical characteristics of raw substrate (RS), substrate from CW receiving urban wastewater (S-UW) and substrate 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.

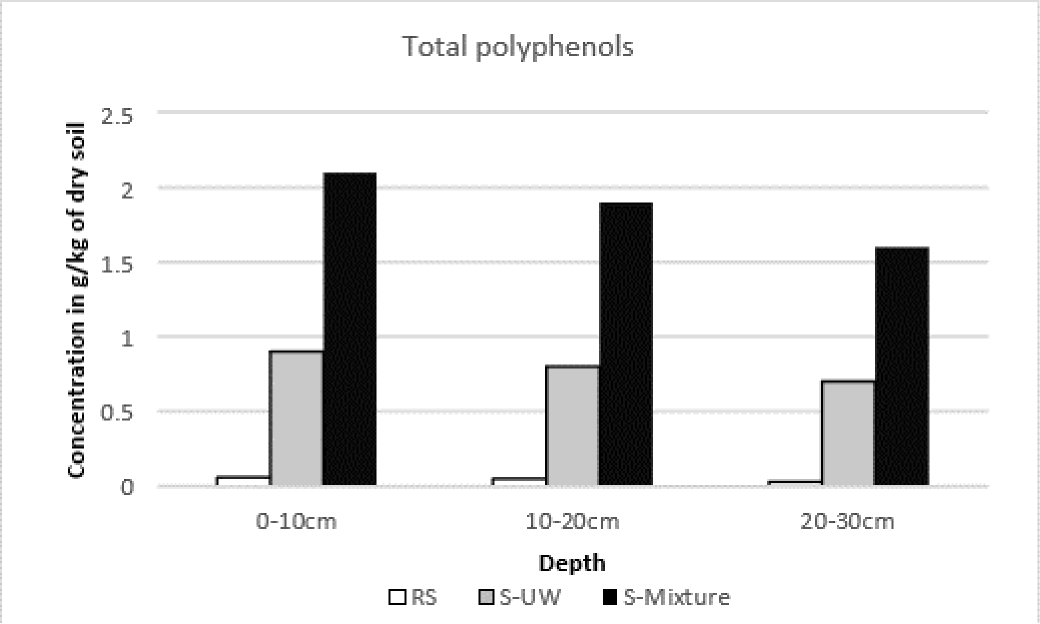

Figure 4

Concentration of total polyphenol in different the substrates

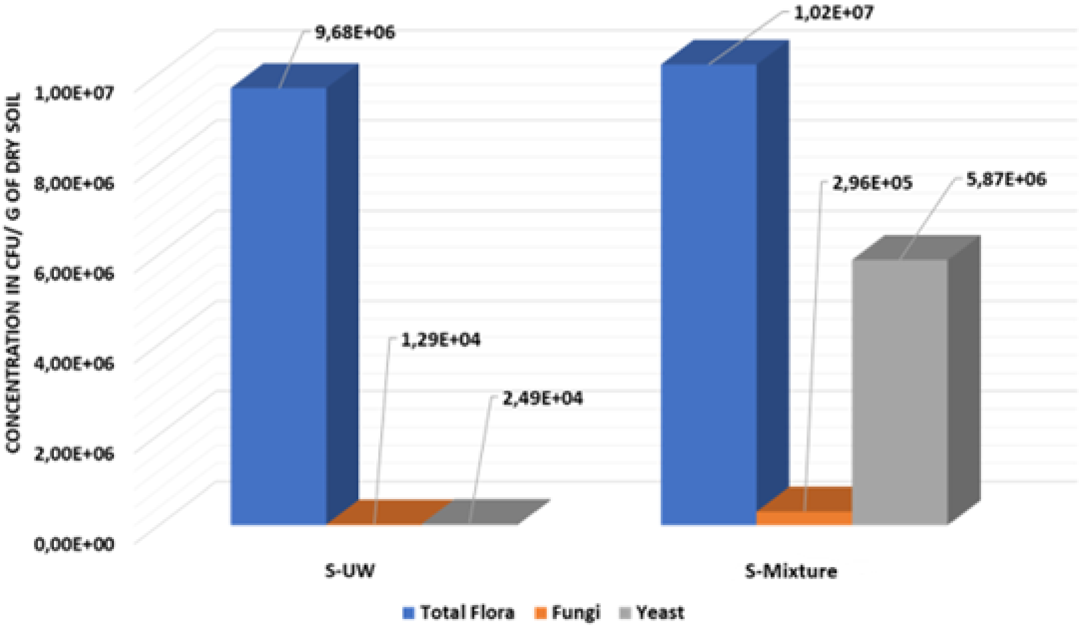

Figure 5

Microbiological characteristics of S-UW and S-Mixture

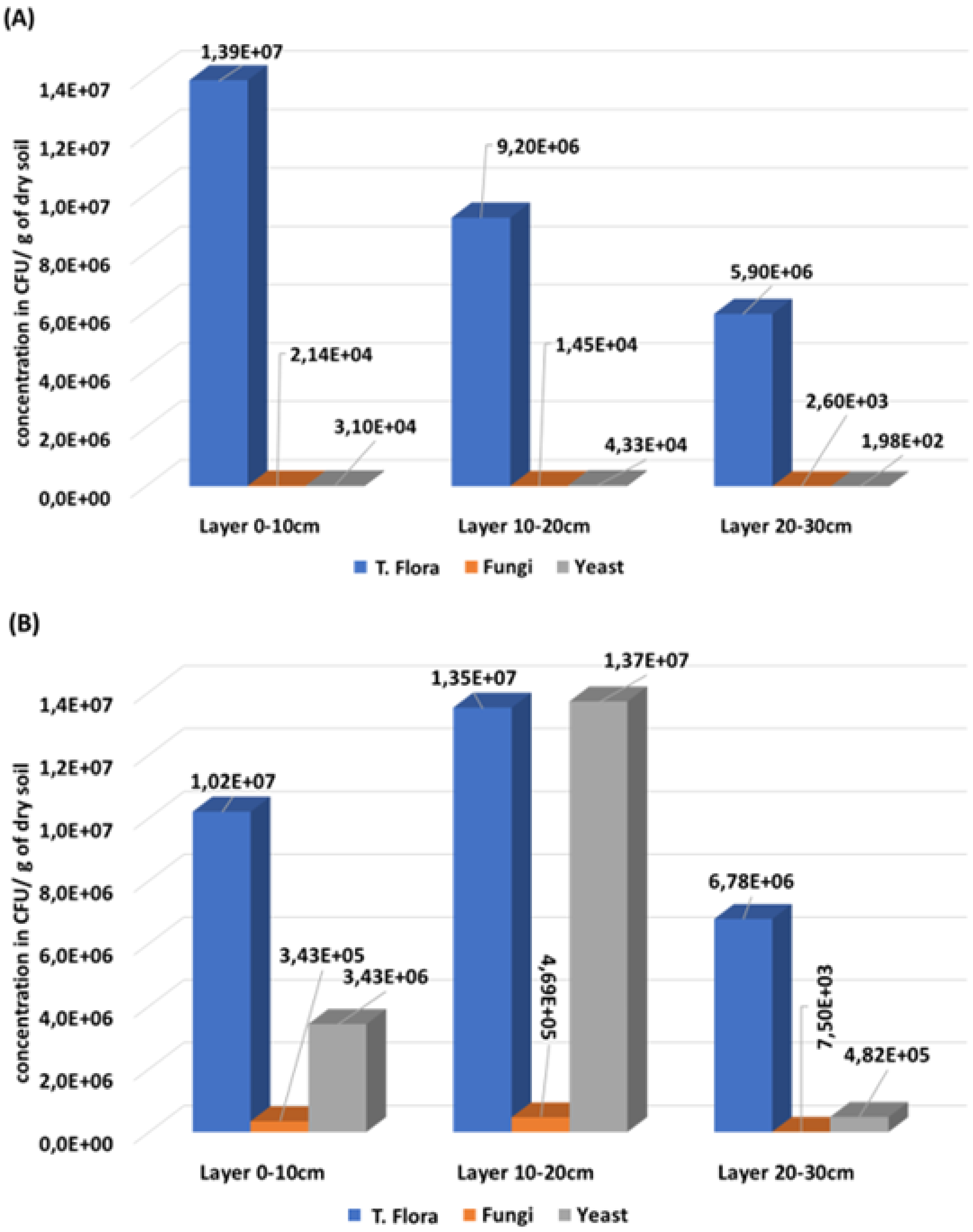

Figure 6

Evolution of Total Flora, fungi and yeast in (A) S-UW and (B) S-Mixture at different depths