Research article

Microbial communities of the olive mill wastewater sludge stored in evaporation ponds: The resource for sustainable bioremediation

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ABSTRACT

Olive Mill Wastewater (OMW) is a polluting residue from the olive oil industry. It is usually stored in open-air unprotected evaporation ponds where their sediments accumulate. This study compares the characteristics of OMW sludges stored for long-time in evaporation ponds and assesses their impact on the underlying soil layer. Physicochemical parameters, toxicity bioassays, and full characterization of the microbial community were analyzed. The extension of the polluting effects was assessed by analysis of toxicity, microbial biomass carbon, and respiration. Geostatistics was used to predict their spatial distribution. Organic matter and polyphenol content besides toxicity levels determine variations between OMW sludges and have a high impact on the microbiota they contain. The microbial community was abundant, diverse, and functionally active. However, the biodegradability of the sludges was hindered by the toxicity levels. Toxicity and biomass carbon were higher on the surface of the ponds than in the soil layer revealing a reduced leach flow and depletion of contaminants. The natural microbiota might be biostimulated by means of applying sustainable and feasible biological treatments in order to favor the OMW sludges bioremediation. These results open up the possibility of solving the environmental concern caused by its storage in similar scenarios, which are common in olive oil-producing countries.

1. Introduction

The olive oil-processing industry has prominent socioeconomic importance that is progressively growing. The average yearly production of olive oil worldwide is around 3.4 million tons, 98% of which are produced in the Mediterranean basin (Souilem et al., 2017). This agro-food sector generates large amounts of solid and liquid residues that should be properly managed to avoid environmental contamination. The characteristics and type of these residues greatly depend on the olive oil extraction system used (Kavvadias et al., 2017). Traditional press and three-phase decanter (3P-D) systems produce two residues: liquid olive mill wastewater (OMW) and solid olive pomace (OP). The 3P-D was introduced in the 70s in the olive oil industry and it was widely adopted up to the 90s when a more efficient olive oil extraction process was developed, consisting of a two-phase decanter (2P-D). This system produces only one semisolid residue as a mixture of the liquid and solid

fraction of the olive. Despite the 3P-D is not used as much as before, it is still employed in many countries and large quantities of over 30 million m^3 of OMW are generated per year, whose management remains a challenge today (Souilem et al., 2017).

OMW is a liquid emulsion with a strong odor and dark brown color, which is composed of 50–94% water, 0.4–2.5% mineral salts, and 4–16% organic matter (Paredes et al., 1999). Regarding the organic fraction, phenolic compounds (2–5%) constitute the most recalcitrant fraction of this effluent, and they are also the main responsible for the antimicrobial and phytotoxic effects of OMW (Babić et al., 2019). These phenolic compounds include simple molecules such as phenolic acids, i. e. tyrosol or hydroxytyrosol, and highly polymerized compounds such as oleuropein (Dermeche et al., 2013).

The most common method for the OMW disposal has been its accumulation in open-air shallow evaporation ponds where the water evaporates during the hottest period of the year and the OMW sediments

remain as a sludge up to the next olive harvest season when the ponds are filled again with OMW (Kavvadias et al., 2017). The number of OMW evaporation ponds throughout the Mediterranean basin has increased over time because this disposal method has been widely implemented (Jarboui et al., 2010; Komnitsas et al., 2016). However, it has many drawbacks, the most important being the contamination of the surroundings of the ponds, i.e. soil and groundwater (Kavvadias et al., 2017). Most research dealing with OMW sludge characterization and its impact in the surrounding soils focus on the chemical and physico-chemical analysis (Abid et al., 2006; Kavvadias et al., 2017; Komnitsas et al., 2016) while the biotic component is scarcely considered. Such studies are important since they provide valuable information that would help to design future *in situ* bioremediation strategies. Recent studies proposed the treatment of OMW sludges in the corresponding ponds by composting (Martínez-Gallardo et al., 2020) or vermicomposting (Sanchez-Hernandez et al., 2020). The correct application of such techniques requires the knowledge of both the characteristics of the OMW sludges, especially the potential response of the biotic component, and the analysis of the extent of the contamination produced by them when stored in unprotected ponds.

In this study, we compare the characteristics of different OMW sludges stored for a long time in non-waterproofed evaporation ponds and assess their impact in the soil beneath the ponds. For these purposes, we focus on the biotic component as the key driver to explain differences among OMW sludges and toxic effects. This study provides one of the more in-deep analysis of the microbial community of such complex environment up to date that will help to design bioremediation strategies in OMW evaporation ponds.

2. Material and methods

2.1. Study area and sampling

The study was performed in OMW evaporation ponds located in the municipality of Mora (Toledo, Spain) (39°40′ 07.4″ N 3°49′ 40.2″ W). The OMW disposal facility covers a total area of 52,440 m^2 and includes eight open-air evaporation ponds (Fig. 1). This facility was used for the disposal of OMW from 1982 to 2007, when it was closed down. The evaporation ponds were constructed by using native soil and simple engineering without impermeable membranes or other protective media, except for the most recently built pond 8 (Fig. 1). The ponds are connected by underground channels, which allowed OMW drainage
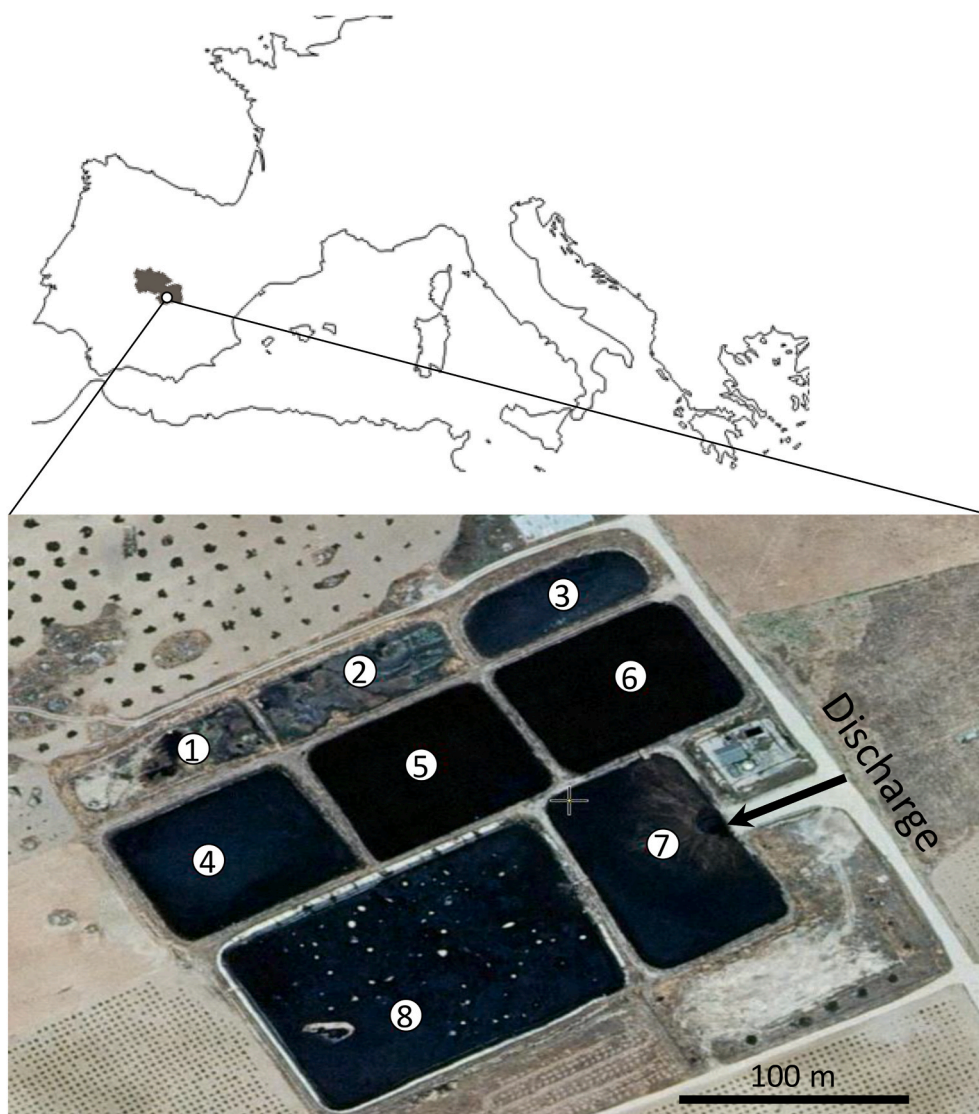

Fig. 1. OMW evaporation ponds located in Mora Municipality (Toledo, Spain). The area includes eight evaporation ponds that are numbered. The study focuses on ponds 1 to 7 that contain OMW sediments/sludge. Pond 7 was the OMW discharge pond. The surfaces are as follows: 2400 m^2 Ponds 1 to 3; 4600 m^2 Ponds 4 to 6; and 5500 m^2 Pond 7. (Modified from Google Image).

from the discharge point in pond 7 to the other ponds based on flow. Currently, only the waterproofed pond 8 has liquid OMW while the other ponds contain OMW sludges having different levels of moisture. The study was focused on ponds filled with OMW sludges, i.e. ponds 2 to 7. Pond 1 mostly held sandy soil material with no sludge on it but just one small spot of concentrated sticky OMW at the pond border. A sample from this concentrated OMW was taken exclusively for microbial community and toxicity effects analysis as the reference of the harshest conditions. For the analysis of OMW sludge, representative composite sludge samples (~5 kg) were taken from four to eight different points of the ponds at two depths, covering nearly the total sludge column length. These were pooled, thoroughly mixed, and split into three replicates that were subjected to analysis.

A detailed analysis of the distribution of sludge characteristics at surface and subsurface up to the soil level was performed in pond 2. Samples (~500 g) were collected from a grid of 18-points of the OMW sludge layer (0–45 cm) and the soil layer underneath (45–90 cm). An integrated sample of agricultural soil taken from 20 points located in two concentric circles of 100 m and 250 m from the evaporation ponds was used as a reference (undisturbed soil) and named as surrounding agricultural soil (SAS).

All samples were kept refrigerated during transportation to the laboratory. Fresh samples were used for microbial counts, moisture, and pH analysis. For the other analytical methods, the samples were stored at $-20\,°C$ or $-80\,°C$ in the case of metagenomics, until analysis. All results were expressed on a dry weight basis.

2.2. Analytical methods

2.2.1. Chemical and physicochemical analysis

The dry matter of the samples was determined after 24 h at 105 °C. The total organic matter content (OM) was determined by weight loss on ignition at 550 °C for 3.5 h pH and electrical conductivity were analyzed in a 1:10 (w/v) water extract. Polyphenolic compounds were measured at 765 nm using the Folin-Ciocalteu (Beltrán et al., 1999) with Gallic acid standard.

2.2.2. Phytotoxicity and ecotoxicity analysis

Phytotoxicity was evaluated by the analysis of germination of *Lepidium sativum* seeds, according to Zucconi et al. (1985) with slight modifications. Briefly, a 1/10 (w/v) filtered (0.45 μm) aqueous extract (4 mL) was added to filter paper in 12×12 cm sterile square plates and 25 seeds of *L. sativum* were placed in the filter. Plates with distilled water were used as control. For each sample, four replicate plates were used. After incubation at 25 °C for 48 h in the dark, the number of germinated seeds and the length of the root were measured. The Germination Index (GI) was expressed as percentage (GI %) with respect to the control as follows: GI = [(Gs % x Ls)/(Gdw % x Ldw)] x 100. Where: Ls: Length of roots (mm) in presence of the sample; Gs: Number of seeds in the presence of the sample that germinated; Ldw: Length of roots (mm) in seeds treated with water (control); Gdw: Number of seeds treated with water (control) that germinated.

Ecotoxicity was determined using the bioluminescence inhibition test of the bacterium *Aliivibrio fischeri* (formerly *Vibrio fischeri*) according to Jarque et al. (2016). The Aboatox kit (kit 1243-500 BioTox; Aboatox, Finland) was used following the protocol established for the reconstitution of *A. fischeri*. Luminoskan ascent luminometer (Thermo Fisher Scientific, USA) was used for luminescence measurement. The concentration of the sample that causes a 50% decrease in the light emitted by the *A. fischeri* (EC50) was calculated using regression equations. Finally, the toxicity units (TU) were calculated according to the expression TU = [1/(EC50)] × 100.

2.2.3. Microbial analysis: counts, taxonomic and functional biodiversity

Total and polyphenol-degrading culturable bacteria and fungi were quantified. Ten-fold serial dilutions were prepared from a 1/10 (w/v)

dilution of fresh sample in sterile saline solution (0.9% w/v NaCl) and spread out on specific culture media for each microbial group. Plate Count Agar (PCA) (Panreac, Spain) and Rose Bengal Chloramphenicol Agar (RB) (Panreac, Spain) were used for total bacteria and fungi colony counts, respectively. Polyphenol-degrading microorganisms were count as colonies surrounded by a brown halo in tannic acid (Sigma) (0.5% w/v) supplemented PCA and Potato Dextrose Agar (PDA) (Panreac), for bacteria and fungi respectively. All plates were incubated at 30 °C for 48 h (bacteria) or 96 h (fungi). Colony forming units (CFU) were count and the results were expressed as log CFU g^{-1} dry weight.

The taxonomic bacterial and fungal biodiversity was carried out by metagenomic analysis. DNA from samples (~1 g) was extracted by using the DNeasy PowerSoil DNA isolation kit (Qiagen, Germany). For fungi, the complete ITS2 region (around 300 bp) was amplified using the primers ITS86F (5′ GTG AAT CAT CGA ATC TTT GAA 3′) and ITS4 (5′ TCC GCT TAT TGA TAT GC 3′). For bacteria, a fragment of the bacterial 16S rRNA gene (around 460 bp) was amplified using the primers Bakt 341F (5′ CCT ACG GGN GGC WGC AG 3′) and Bakt 805R (5′ GAC TAC HVG GGT ATC TAA TCC 3′). In both cases, the Illumina primer sequences were attached to their 5′ ends. PCRs were carried out with 2.5 μL DNA template, 0.5 μM primers, 12.5 μL Supreme NZYTaq 2x Green Master Mix (NZYTech, Portugal), and ultrapure water up to 25 μL. The PCR reaction was performed as follows for fungal DNA: initial denaturation at 95 °C for 5 min, followed by 35 cycles of 95 °C for 30 s, 49 °C for 30 s, 72 °C for 30 s, and a final extension step at 72 °C for 10 min. The PCR for bacterial DNA was as follows: initial denaturation at 95 °C for 5 min, followed by 25 cycles of 95 °C for 30 s, 50 °C for 30 s, 72 °C for 30 s, and a final extension step at 72 °C for 10 min. The oligonucleotide indices required for multiplexing different libraries in the same sequencing pool were attached in a second PCR round with identical conditions but only 5 cycles and 60 °C as the annealing temperature. Libraries were purified using the Mag-Bind RXNPure Plus magnetic beads (Omega Biotek, USA). Then, they were grouped in equimolar amounts and sequenced in a MiSeq PE300 (Illumina, USA) by AllGenetics (La Coruña, Spain).

Functional biodiversity was determined by analyzing the physiological profile at the community level using Biolog EcoPlate ™ microplates (Biolog, USA) according to Feigl et al. (2017) with some modifications. Fresh sample (2 g) was suspended in 18 mL sterile saline solution and diluted up to 10^{-3} under sterile conditions. From this dilution, 150 μL were pipetted into each well of the Biolog EcoPlate™ microplate and incubated at 25 °C for 48 h. After incubation, the optical density (OD) at 590 nm was determined by EON microplate spectrophotometer (Biotek, USA). The optical density or color development (ODi) of each well was corrected with the control well (water). The corrected ODi values and number of substrates N = 31 were used to calculate: functional richness (R = sum of the number of cells where ODi≥0.15); functional activity intensity as Average Well Color Development (AWCD = ΣODi/N); functional biodiversity as Shannon index (H' = −Σpi ln(pi), where pi = ODi/ΣODi), and Shannon evenness (E = H'/lnR). The results were also expressed as substrate average well color development (SAWCD) for each of substrate categories (Sala et al., 2010).

2.2.4. Respiration indices and microbial biomass carbon

The respiration indices were determined according to Adani et al. (2006) by using the respirometer described by Ponsá et al. (2010). The analysis was performed by incubating 150 g of sample (60% moisture) in a 500 mL bottle in a water bath at 37 °C for 4 days. A constant airflow was supplied and the oxygen in the exhaust gas was measured. Two respiration indices were calculated according to Ponsá et al. (2010): Dynamic Respiration Index (DRI) and Cumulative Oxygen Consumption (AT4). DRI, expressed as g of oxygen consumed per kg of organic matter (OM) and per hour (g O_2 kg^{-1} OM h^{-1}), was calculated from the average value of oxygen consumed during 24 h of maximal biological activity. AT4, expressed as g of oxygen consumed per kg of organic matter (g O_2

kg^{-1} OM), was determined from cumulative oxygen consumption over four days after overcoming the lag phase. Microbial biomass carbon (Cbio) was evaluated by the fumigation-extraction method according to Vance et al. (1987).

2.3. Bioinformatics and data analysis

For metagenomic data analysis, the Illumina paired-end raw data contained the demultiplexed FASTQ files, i.e. forward (R1) and reverse (R2) reads with their quality scores sorted by sample. The indices and sequencing primers were deleted during the demultiplexing step. The quality of the demultiplexed FASTQ files was verified by FastQC software (Andrews, 2010). Paired-end assembly of the forward and reverse reads was performed with FLASH. CUTADAPT software 1.3 was employed to delete sequences below 300 nt that were not in the PCR. FASTQ files were checked using QIIME v1.9.0. The sequences were quality-filtered with a Phred score of 20 quality threshold and the Chimeric sequences were deleted using the UCHIME algorithm implemented in VSEARCH, with a reference based on chimera detection using Silva Database (Quast et al., 2013). The sequences were taxonomically assigned using the open-reference approach in QIIME. The operational taxonomic units (OTU) picking was run using the Silva Database and each OTU was assigned to a microbial taxon using the UCLUST algorithm with a confidence threshold of 97%. Singletons and OTUs with less sequence than 0.005% of the total were excluded from the analysis. OTUs of each sample with low abundance (below 0.1%) were removed to correct the index-jumping phenomenon. Alpha diversity indices (i.e., ACE, Chao1, Shannon, and Simpson) were calculated from rarefied samples using QIIME.

For the analysis of physicochemical, toxicity, and microbiological data, all measurements were carried out at least in triplicate, and data are presented as the mean. The Shapiro-Wilk test was performed for normality testing of the datasets. Box-Cox transformation was performed to ensure the assumption of parametricity when required, the transformed data were converted using back transformation into the original values for representation. The significance level of $p < 0.05$ was used for all statistical analyses. Analysis of variance (ANOVA) and post hoc multiple comparison tests (Least Significant Difference Fisher Test) were performed to compare mean values for the different factors analyzed. To identify the relationship between physicochemical, toxicity, and microbiological parameters, correlation analysis and principal component analysis were performed. Geostatistical mapping was performed using the method of ordinary kriging with an omnidirectional variogram that was used to construct spatial distribution maps in the OMW sludge and soil layers. All data analyses were performed using Statgraphics Centurion 18 (StatPoint Technologies Inc., Virginia, USA).

3. Results and discussion

3.1. Characteristics of OMW sludges

3.1.1. Physicochemical parameters

Table 1 shows moisture, pH, electrical conductivity (EC), organic matter (OM), and total polyphenol content of OMW sludges from the ponds 2 to 7. Pond 1 was excluded for these analyses because it lacked the sludge layer. According to moisture, pH, and OM content, there were two types of OMW sludges: mineral alkaline dry sludges from ponds 2 and 3, and organic neutral moist sludges from ponds 4 to 7. These differences are likely due to the suspended material stratification when the evaporation ponds were active (Fig. 1). Ponds nearby discharge point (ponds 4 to 7) received more OMW sediment and thus have more organic matter than the ponds faraway (ponds 1 to 3). Besides, mineral materials from ponds 2 and 3 could have been subjected to lesser OMW filling events and more mineralization of sludge than the material from the other ponds. This may explain the alkaline values of sludge from these ponds, which were far higher than the pH values of fresh OMW, around 4–5.5 (Paredes et al., 1999). It is known that the mineralization of OMW leads to an increase of pH (Kavvadias et al., 2017). Also, it can be a consequence of sludge amendments with calcium or sodium hydroxide, which is a common practice to ameliorate odor release from ponds (Ntougias et al., 2013). Other parameters that differentiate the OMW sludges were EC and polyphenol content. The material from the discharge pond 7 together with those of ponds 2 and 3 had low values of both EC (2–5 mS cm^{-1}) and polyphenol content (5–21 g kg^{-1}) in comparison to those of ponds 4 to 6 (EC 13–15 mS cm^{-1} and polyphenol content 38–49 g kg^{-1}). Fresh OMW has EC usually high (around 13 mS cm^{-1}) (Haouache and Bouchaleta, 2016) and its polyphenols content ranges between 0.1 and 4 g kg^{-1} (Babić et al., 2019). The low values of EC in ponds 7, 2, and 3 could be explained by a washing effect upon rainfall episodes combined with the dynamic of water flow in the ponds (Kavvadias et al., 2017). Total phenols in fresh OMW are far lower than the values obtained in this work for most of the sludges with high moisture and organic matter load, which were enriched in those compounds. This supports the fact that recalcitrant compounds would concentrate over time in stored OMW sludge.

3.1.2. Toxicity

In general, the toxicity of sludges both on plants (phytotoxicity) or microorganism model *A. fischeri* (ecotoxicity) followed a similar pattern (Fig. 2). According to them, the samples can be classified into three levels of toxicity: highly toxic (ponds 1, 4, and 5), mderately toxic (ponds 2 and 3), and low toxic (ponds 6 and 7). The average TU value was 35 and oscillated from 9 in pond 6 to values higher than 50. These results agree with previous studies on fresh OMW toxicity using the same bioassay (Amaral et al., 2012; Babić et al., 2019). The phytotoxicity was also variable among the sludges, but all exhibited phytotoxic values. The lowest GI value (20%) (higher phytotoxicity) was obtained in the sample taken from pond 1, that together with sludges from ponds 4 and 5 (GI>50%) gave GI values considered highly phytotoxic.

Table 1
Physicochemical characteristics of OMW sludges from the evaporation ponds.

Sample (Pond)	Moisture (%)		pH		Electrical conductivity (mS cm^{-1})		Organic matter (%)		Polyphenol content (g kg^{-1})	
2	18.2	a	9.7	a	2.7	a	12.7	a	4.68	a
3	16.1	a	9.5	a	5.3	b	38.5	b	20.67	b
4	49.7	c	6.5	b	13.3	c	69.2	c	43.31	c
5	55.7	c	7.1	b	12.9	c	81.6	d	38.44	c
6	40.3	b	6.9	b	15.3	c	66.6	c	49.41	c
7	54.5	c	6.7	b	5.3	b	81.9	d	9.90	a
	***		***		***		**		***	
LSD	7.8		0.6		1.7		6.7		3.1	

Average values (n = 3) within columns with different letters denote a statistically significant difference based on Fisher's LSD paired post hoc comparisons at 95% confidence level. LSD (Least Significant Difference) interval and significance of ANOVA test ***$p < 0.001$, **$p < 0.01$, *$p < 0.05$ are shown.
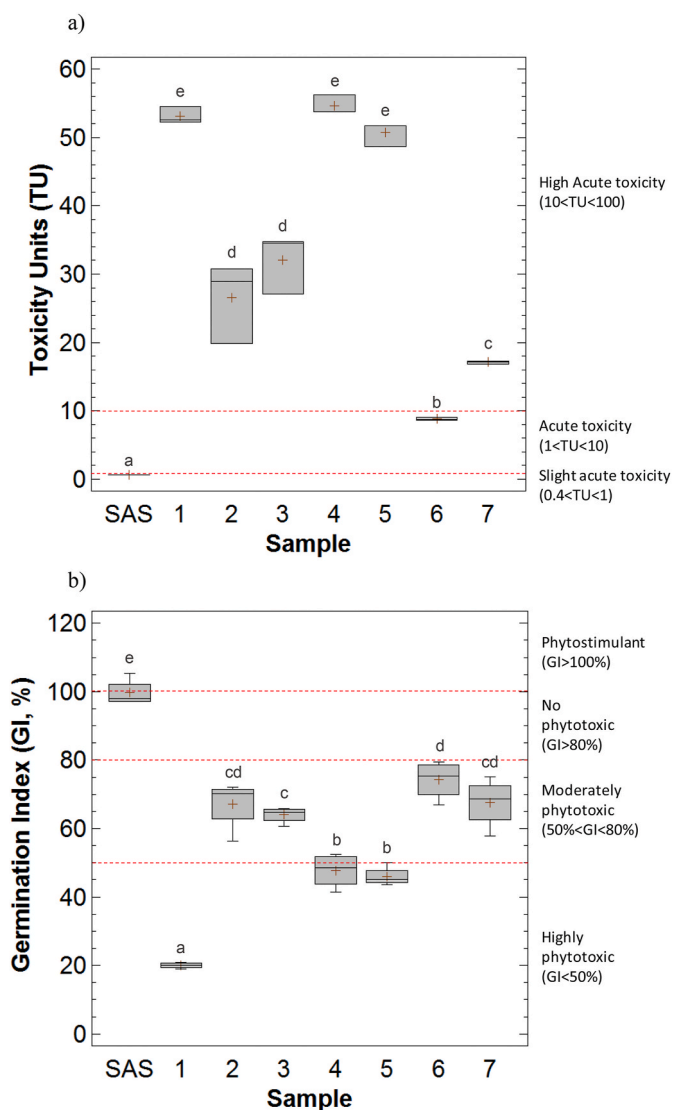
Fig. 2. Boxplot showing a) ecotoxicity, represented as toxicity units (TU = 100/EC50) and b) phytotoxicity, represented as Germination Index (GI %), of OMW sludge samples from evaporation ponds 1 to 7, and surrounding soil sample (SAS) taken as reference. Threshold values and classification are noted with dashed red lines and text on the right for ecotoxicity (Persoone et al., 2003) and phytotoxicity (Zucconi et al., 1985) classification. The values within the box (+) are the average of three replicates. The boxes bearing different letters were significantly different according to Fisher's LSD test (p < 0.05). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

Polyphenol compounds content in addition to high salinity (EC) influence OMW toxicity (Babić et al., 2019). In fact, samples with the lowest CE and polyphenol content from ponds 2, 3, and 7 were the less toxic (lower UT and higher GI values) while those from ponds 4 and 5 that had high CE and polyphenol content showed high toxicity levels. However, that was not the case for OMW sludge from pond 6, which although having the highest EC and polyphenol content, was even less toxic than those from ponds 2 and 3. This indicates that toxicity is much more complex than previously considered and analyzing phenolic compounds and EC, though being good indicators of the potential toxic effect of materials, are not by themselves good enough to predict the impact of a specific OMW sludge.

3.1.3. Microbial load, biodiversity, and metabolic activity

The culturable fungal and bacterial populations in most sludges were

high, with levels for bacteria in the range of 10^5–10^8 CFU g^{-1} and those for fungi 10^3–10^7 CFU g^{-1} (Fig. S1, Supplementary material). Bacteria quantitatively and qualitatively dominated the microbial community over fungi (Table 2; Fig. S1, Supplementary material). These results contradict previous reports that found a predominance of fungi versus bacteria in OMW sludge stored in evaporation ponds or soil amended with OMW (Jarboui et al., 2010; Sampedro et al., 2009). This effect has been attributed to the higher capability of fungi to adapt to recalcitrant compounds and low levels of water activity (Jarboui et al., 2010) as well as to resist the antimicrobial effect of phenolic compounds (Amaral et al., 2012). These conditions would occur in OMW sediments during storage in evaporation pond. However, their effect on shaping the microbial community could be more complex than previously stated. In addition, it has to be considered that upon long-term exposure to stressful conditions, the microbial population adapts. In fact, it was clear that the microbial community was enriched in specialized representatives as evidenced by the high proportion of polyphenol-degrading (PO) bacteria and fungi in most ponds (Fig. S1, Supplementary material). In pond 6, whose OMW sludge had the highest amount of polyphenols recorded (Table 1), 100% of the culturable fungal population had polyphenol oxidizing activity. Fungi are characterized by the production of enzymes that provide them with degrading activity and tolerance to high concentrations of polyphenolic compounds (Jarboui et al., 2010). But this capability is not restricted to fungi, many bacteria are capable of using polyphenol compounds (Pepi et al., 2010). Consequently, in an aged OMW sludge, the bacterial population could adapt just as well as the fungal population, even exceeding it in microbial load. This was noted also by the biodiversity differences (Table 2). As revealed by the metagenomic analysis, the total number of reads obtained from the seven OMW sludge samples was 149,106 for bacterial 16S rRNA and 205,594 for fungal ITS. These clustered into 905 and 811 OTUs, respectively (Table 2). Shannon biodiversity H′ average values of 3 for bacteria and 1.5 for fungi, can be considered moderately high (Table 2). In general, materials from ponds 4, 5, and 6 had less rich and diverse bacterial communities than the other sludges. In the case of fungi, as predicted, the sample from pond 1 stood out for its extremely poor fungal community, having Shannon and Simpson indices close to zero, which is indicative of a low number of species with a few representatives. In contrast, the sample from pond 5 had the highest fungal

Table 2
Richness and diversity estimation of the 16S rRNA/ITS (bacterial/fungal) sequencing libraries from the MiSeq sequencing analysis in OMW sludges from evaporation ponds 1 to 7.

Sample (pond)	No of reads	Richness (OTUs Obs)	Chao_1	Shannon	Simpson (1-D)
Bacteria (16SrRNA)					
1	25,292	94	104	3.15	0.93
2	25,488	182	214	3.19	0.87
3	5823	164	187	3.52	0.93
4	36,169	92	102	2.89	0.89
5	22,771	99	107	2.35	0.78
6	15,143	118	180	2.76	0.87
7	18,420	156	177	3.74	0.94
Fungi (ITS2)					
1	14,936	6	7	0.04	0.01
2	18,348	26	29	1.57	0.52
3	20,543	20	22	1.30	0.36
4	34,449	11	17	1.27	0.48
5	34,955	40	52	2.53	0.76
6	39,744	22	28	1.20	0.40
7	42,619	27	32	1.01	0.26

The cutoff value is 0.03 (sequence identity 0.97). OTUs and Chao are used to evaluate the community richness. Shannon and Simpson corrected are used to assess the community diversity and evenness. All results are based on a rarefied depth of 5500 sequences for bacteria and 14,000 sequences for fungi. Average goods coverage was 99%.

richness, biodiversity, and evenness values.

The structure of the bacterial and fungal community according to genus diversity is shown in Fig. 3. The bacterial community was dominated by the phylum *Proteobacteria* followed by *Actinobacteria* (Fig. S2, Supplementary material), as it has been also found in other OMW-related environments (Hernandez et al., 2018; Ntougias et al., 2013). However, there were slight differences at the phylum level between ponds that could be ascribed to physicochemical and nutrients availability differences. Thus, *Actinobacteria* was the dominant phylum in the mineral dry alkaline ponds 2 (58%) and 3 (45%); *Proteobacteria* in the ponds with higher moisture, organic load and neutral pH (Ponds 4 to 7 with levels higher than 53%), and *Firmicutes* in the sample from pond 1 (59%) having extreme conditions (Fig. S2, Supplementary material). Noteworthy, the most ubiquitous bacterial genus was *Halomonas*, a halotolerant member of the phylum *Proteobacteria*, which is mostly isolated from saline environments around the world (Vreeland, 2015), and reported in OMW environments (Ntougias et al., 2013). In line with its tolerance to salts concentration, this bacteria accounted for more of 50% of the bacterial sequences in the ponds with higher EC (Ponds 4 to

6), and also in the sample from pond 1, the concentrated OMW sample; while its levels were low or null in ponds having lower EC (Ponds 2, 3, and 7). Another halotolerant bacteria found in most ponds was *Alkalibacterium*, a member of the phylum *Firmicutes*, which is an obligate alkaliphilic requiring pH above 8 to growth (Ntougias and Russell, 2015) and, as such, it was one of the main representatives in pond 1 and alkaline sludges from ponds 2 and 3. Other bacterial genera widely present in most ponds were *Paracoccus*, also found earlier in OMW (Ntougias et al., 2013), and *Nocardiopsis*, a member of the phylum *Actinobacteria* not reported earlier in OMW. In contrast to the common distribution of bacteria among different ponds, the fungal community was dominated in each pond by a particular genus as it was indicated by the low evenness values (Table 2). The fungal community included mainly members of the phylum *Ascomycota* (80%) while representatives of the phylum *Basidiomycota* accounted for the 20% (Fig. S2, Supplementary material). The dominant fungi for each pond were *Fusarium* (67%) in pond 2, *Aspergillus* (80%) in pond 3, *Scopulariopsis* (68%) in pond 4, *Tritirachium* (40%) in pond 5, *Scedosporium* (76%) in pond 6, and *Microascus* (93%) in pond 7. These fungal genera have been earlier
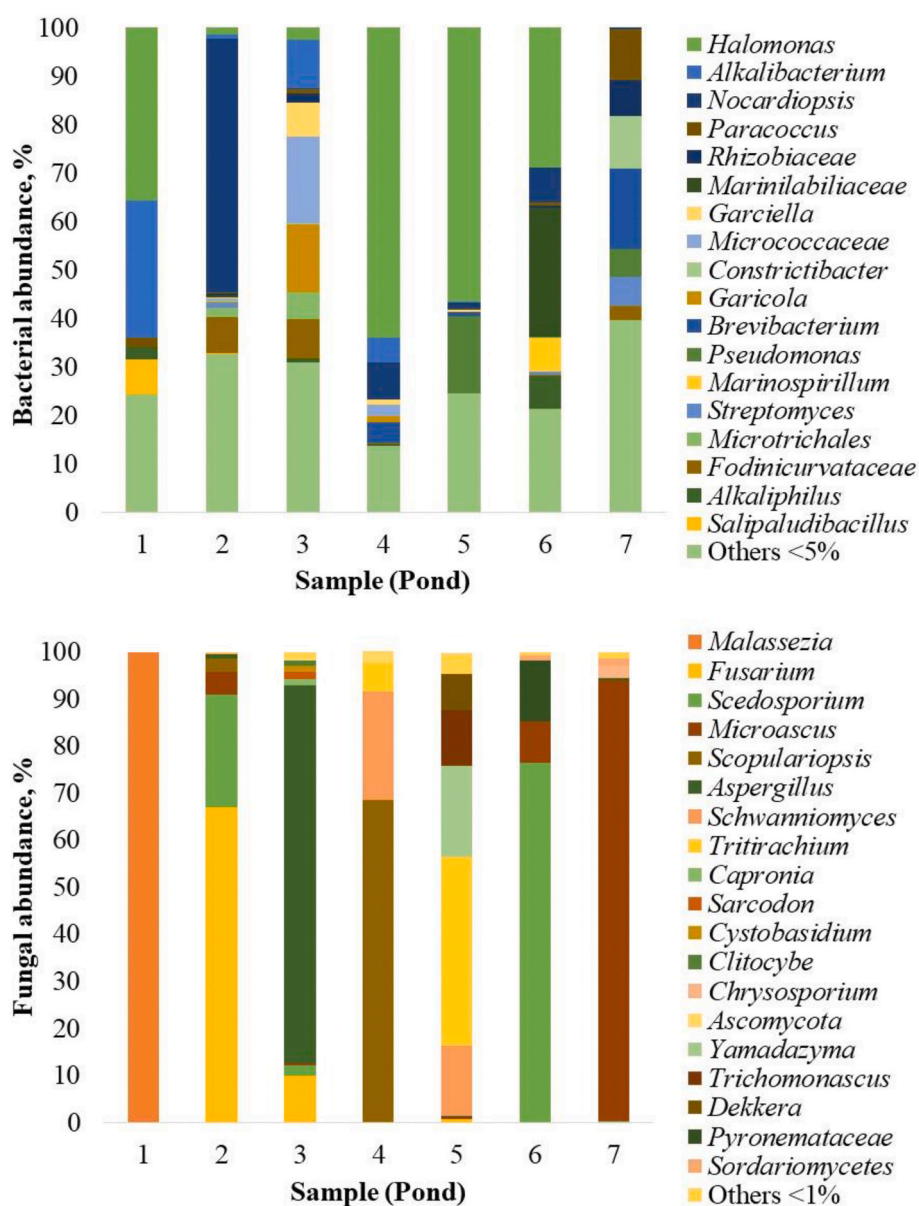

Fig. 3. Bacterial and fungal genera diversity expressed as relative abundance (%) of OTUs in OMW sludge samples from evaporation ponds 1 to 7. For uncultured or ambiguous taxa sequences the lower known taxonomic rank is indicated as unclassified.

described in OMW-related environments (Hernandez et al., 2018; Ntougias et al., 2013). Their success for one specific material in the ponds is a sign of the site-specific adaptation. The extreme case is the fungal community of sludge from pond 1, where a single fungal genus identified as *Malassezia* was found, which is described for the first time in OMW. This *Basidiomycete* is a yeast-like genus associated to the skin and mucous membranes of animals, sometimes generating some seborrheic dermatitis (Crespo-Erchiga and Florencio, 2006). To our knowledge, this is the first time this genus is described outside skin-related microbiota.

The functional analysis of microbial communities allows clarifying the interactions between the organisms and the environment. In this work, this analysis was performed using the Biolog Ecoplate technique that is a culture-dependent approach, which reveals the most active populations within the microbial community and thus, the community's physiological patterns (Feigl et al., 2017). The Biolog Ecoplates contain 31 substrates that can be potentially used as nutrients by microorganisms. The number of substrates metabolized and other functional biodiversity indices of the microbial community of the samples are shown in Table 3. The number of substrates oxidized by the OMW sludge community (functional richness) averaged 12 out of the 31 carbon sources, with a minimum of 1 in pond 1 and a maximum of 18 in pond 6. This was also the trend for the intensity of the metabolic activity, as shown by the values of AWCD (Average well color development), that reached the highest value (0.51) in pond 6 and the lowest in pond 1, where a negligible activity was found. The functional biodiversity expressed as Shannon index (H′) was moderately high in all ponds, with a range of values between 2.0 (pond 1) and 2.8 (pond 6); while Shannon Evenness values indicate an unbalanced metabolic profile for the microbial community in pond 1 (low number of activities highly represented) and more balanced in the other samples. These results revealed that the availability of substrates contained in the organic matter and the toxic pressure were the main factors that influence the functional diversity of the microbial community of OMW sludges. Thus, the sample from pond 6 was the least toxic and had the highest intensity and diversity of microbial activity while the opposite occurred for the sample from pond 1.

The relative abundance of the metabolic activities grouped according to the chemical family of the substrates is shown in Fig. 4. The dominant functional activities were those related to the metabolism of polymers (31%) and carboxylic acids (24%), followed by carbohydrates and amino acids, both with average values of 14%, and amines and phenolic acids that were the less represented with relative average abundances of 9 and 7%, respectively. These results reflect that the majority of carbon sources in organic matter stored in the ponds might be recalcitrant
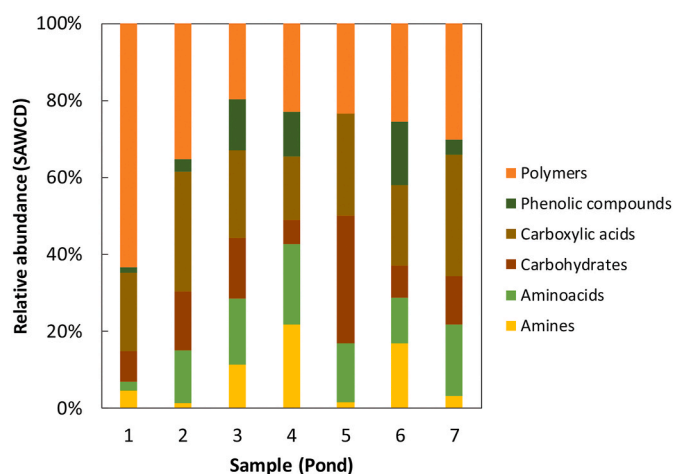

Fig. 4. Relative abundance of metabolic activities according to substrate average well color development (SAWCD) of substrate categories present in Biolog EcoPlates® consumed by microbial communities in the different OMW sludges from ponds 1 to 7. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

polymers whose utilization could be mainly associated with the activity of fungal and Actinobacteria populations. These are known to be the main microbial groups involved in the utilization of polymers because of the production of extracellular enzymes (Kapur et al., 2018). The extreme case was the sample from the pond 1, in which 63% of the overall metabolic activity was related to polymer utilizing activity. This might be due to the scarcity of small sugars and organic acids that forces the utilization of alternative recalcitrant OMW-derived polymeric substrates. The activities related to the metabolism of carboxylic acids and carbohydrates, which are intermediates of organic matter degradation, were well represented in all OMW sludge samples. Those are likely used as the main carbon source for the abundant heterotrophic bacterial community. The fact that the utilization of carboxylic acids was the second largest metabolic activity after that of polymers, may be related to the higher presence of these compounds in the sludges. These are mainly produced by bacteria and yeast during fermentative metabolism. This suggests an important anaerobic metabolic activity in the sludges during the storage. In addition, it is necessary to highlight the existence of a notable presence of activities related to the degradation of phenolic compounds. This was highly variable between the different ponds but somewhat related to the polyphenol content, e.g., sludge from pond 6 had the highest polyphenol content and also the higher metabolic activity for those compounds. Hentati et al. (2016) also found sharp differences in functional diversity between soils taken in the near an OMW evaporation pond with a clear dominium of carbohydrates and carboxylic acids metabolisms.

Finally, it has to be emphasized that despite the toxic pressure recorded, all sludge samples, except that from pond 1, exhibited a wide range of metabolic activities that were also balanced. These represent a pool of potential activities that could be enhanced with the proper management of the environment e.g. supplying fresh organic matter that will have a biostimulant effect.

3.1.4. Relationship between parameters

The correlation matrix among physicochemical, toxicity, and microbiological parameters is shown in Fig. S3, Supplementary material. Moisture correlated negatively with pH ($r = -0.94$) and positively with organic matter ($r = 0.93$), while pH correlated negatively with organic matter ($r = -0.92$). Noteworthy, electrical conductivity and phenolic content showed a strong positive correlation ($r = 0.98$). Moreover, as could be expected, GI and TU negatively correlated ($r = -0.97$), while this latter also correlated negatively with functional

Table 3
Richness and diversity estimation of the physiological profile at the community level from the Biolog Ecoplate analysis of OMW sludge samples from evaporation ponds 1 to 7.

Sample (Pond)	R		AWCD		H′		E	
1	1	a	0.01	a	2.06	a	0.00	a
2	14	c	0.37	e	2.64	de	0.99	b
3	12	b	0.34	cd	2.40	c	0.97	b
4	13	bc	0.36	de	2.48	cd	0.97	b
5	12	b	0.32	c	2.38	b	0.96	b
6	18	d	0.51	f	2.82	e	0.97	b
7	11	b	0.21	b	2.65	de	1.11	c
	**		**		***		***	
LSD	2		0.03		0.23		0.06	

* Values (n = 3) within columns with different letters denote a statistically significant difference based on Fisher's LSD paired post hoc comparisons at 95% confidence level. LSD (Least Significant Difference) interval and significance of ANOVA test ***p < 0.001, **p < 0.01, *p < 0.05 are shown. R: number of substrates oxidized (substrate richness). AWCD: Average color development is an index of the total bioactivity; H′: Shannon index (H) is the functional biodiversity index; E: Shannon evenness.

biodiversity index H' (r = −0.84). Total culturable bacteria and fungi correlated positively (r = 0.90) while the latter was negatively correlated with H' (r = −0.83). Thus, fungal abundance influences negatively the metabolic diversity.

For the PCA analysis, a parameter was selected in many cases between each pair that showed a strong linear correlation. The results of the PCA indicate that PC1 explained 49%, and PC2 27% of the total variance (Fig. 5). PC1 separated the OMW sludges according to their toxicity, which is related to organic matter and phenolic compounds content. The toxicity affects microbial communities by promoting the fungal biodiversity at expenses of bacterial biodiversity and lowering functional diversity. Accordingly, samples from ponds 4 and 5 grouped on the right side of the plot as the most toxic. In addition, PC2 separates OMW sludges based on the functionality of the microbial community being the content of OM and phenolic content the parameters that have also more weight load in determining that functionality. Samples from ponds 4, 6, and 7 exhibited the highest levels in this axis. These results suggest that the organic matter and the polyphenol content besides the toxicity were the main drivers that determine the functional and taxonomical biodiversity of the microbial communities and partitioned the samples accordingly.

3.2. Impact of OMW sludge in soil depth

The spatial distribution of the toxicity (phyto- and ecotoxicity) and Cbio in the OMW sludge and soil layers of the pond 2 is shown in Fig. 6. For comparison, the figure displays the three parameters measured that are represented in boxplots, including means for each layer, together with the spatial distribution of the parameter in OMW sludge (superficial) and soil (deep) layers according to kriging analysis predictions. The

Fig. 5. Biplot of the principal component analysis (PCA) for physicochemical, toxicity and microbiological parameters in the samples from ponds 2 to 7. The two principal components (PC1 and PC2) explained 76%. Arrows represent physicochemical (brown), toxicity (blue), and biological (green) parameters, and circles in grey represent OMW sludge pond sampling points. Variables considered: organic matter (OM), Phenolic compounds (Phen), toxicity units (TU), bacterial sequences Shannon biodiversity index (B-biodiversity H'), fungal sequences Shannon biodiversity index (B-biodiversity H'), and functional Shannon biodiversity index (Func-biodiversity H'). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

respirometry analysis on composite samples of each layer revealed that the respiration (DRI and AT4) was practically null in the OMW sludge layer. In the soil layer, average values of 0.15 g O_2 kg^{-1} OM h^{-1} and 7.8 g O_2 kg^{-1} OM for DRI and AT4 were obtained, respectively. The OMW sludge layer had higher microbial biomass carbon (Cbio) and toxicity than the soil layer (Fig. 6). In general, the toxic effect of OMW sludge did not have a high impact on the soil layer where low ecotoxic and null phytotoxic values were obtained. The spatial distribution of Cbio was quite variable in the OMW sludge layer, in which Cbio sharply ranged from 10 to 535 mg kg^{-1} exhibiting several hot pots mainly in the borders of the pond. In contrast, the values of Cbio were more homogeneous in the soil layer, ranging from 5 to 165 mg kg^{-1}. The ecotoxicity had an average of 22 TU in the OMW sludge layer, and 7 TU in the soil layer. In the OMW sludge layer, the ecotoxicity varied over a wide range (3–55 TU) and the spatial distribution was quite heterogeneous, with an area of the pond showing the highest values that coincide with the area where lower Cbio values were predicted. In the soil layer, most ecotoxicity values were below 10 UT except for two hot spots where it reached 45 TU and 20 TU, respectively. The Germination index (GI%) was higher in the soil layer (110%) than in the OMW sludge layer (72%) and ranged from minor points in the OMW sludge layer having values close to 29% (highly phytotoxic) to the most spread values close to the 72% average. In the soil layer, the values ranged from 66% to 125%, all not phytotoxic, with the lower values (more phytotoxic) obtained in the left upper corner of the pond, which coincides with the area with higher TU values for this layer.

The overall results showed that the toxic effect was limited to the OMW sludge layer while it had a minor impact on the soil layer in the ponds analyzed. In addition, both layers contain a relatively abundant microbial biomass whose natural metabolic activity is restricted. The presence of many hot spots with high Cbio and toxicity values in the surface layer was matched with a dilution gradient in the soil layer. On the other side, the organic matter from OMW is responsible for increases of microbial load but the presence of the toxic compounds inhibits its activity. Therefore, the higher toxicity in the superficial layer inhibits or slows down the metabolic activity of indigenous microorganisms and hence, inhibits respiration avoiding the favorable influence of their higher nutrient content. Previous studies on the effect of soil amendment with OMW or the impact of OMW evaporation ponds report a decrease in organic matter and phenolic compounds with depth (Kavvadias et al., 2017; Komnitsas et al., 2016) and a decline of phytotoxicity of soil as a consequence (Magdich et al., 2012). For these reasons, the native microbial population cannot naturally restore this environment. Although toxic components could reach deep layers by leaching, the impact is lowered by the microbial community that can easily biodegrade them due to the absence of toxic pressure.

4. Conclusions

The microbial community present in OMW sludges is abundant, rich, diverse, and has high metabolic potential but the toxic effects of OMW sludge components limit their natural bioremediation capabilities. These activities could be promoted by biostimulation strategies such as amendment with fresh organic matter. Although the OMW disposal in permeable evaporation ponds has significant impacts on the underlying soil, the contaminating effects are mainly limited to superficial OMW sludge layer. Taking advantage of the biological potential present in the sludges, an *in situ* bioremediation treatment, based on stimulating the growth of the indigenous microbiota, can be limited to the upper layer, without the need to carry out deep excavation operations. The knowledge provided by this study can be extrapolated to similar scenarios in OMW evaporation ponds widely spread throughout the Mediterranean basin and, in addition, contributes to the selection of sustainable and eco-friendly alternatives for the management of this sludge solving this environmental concern.
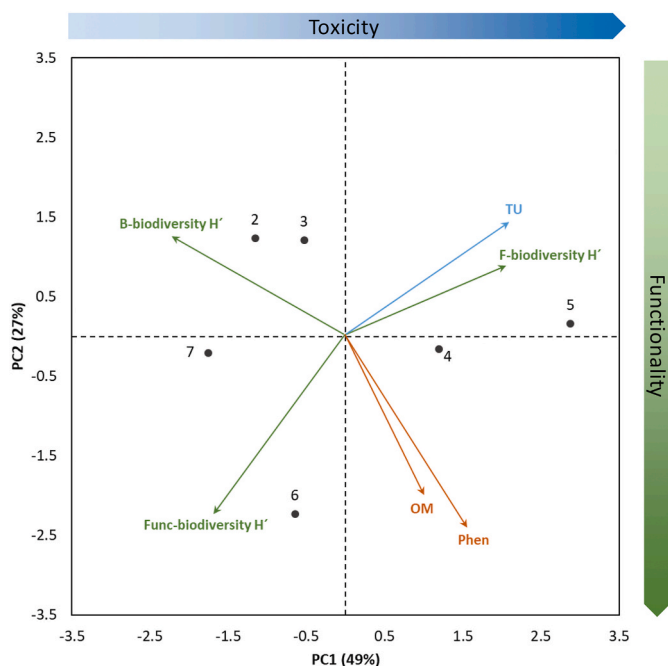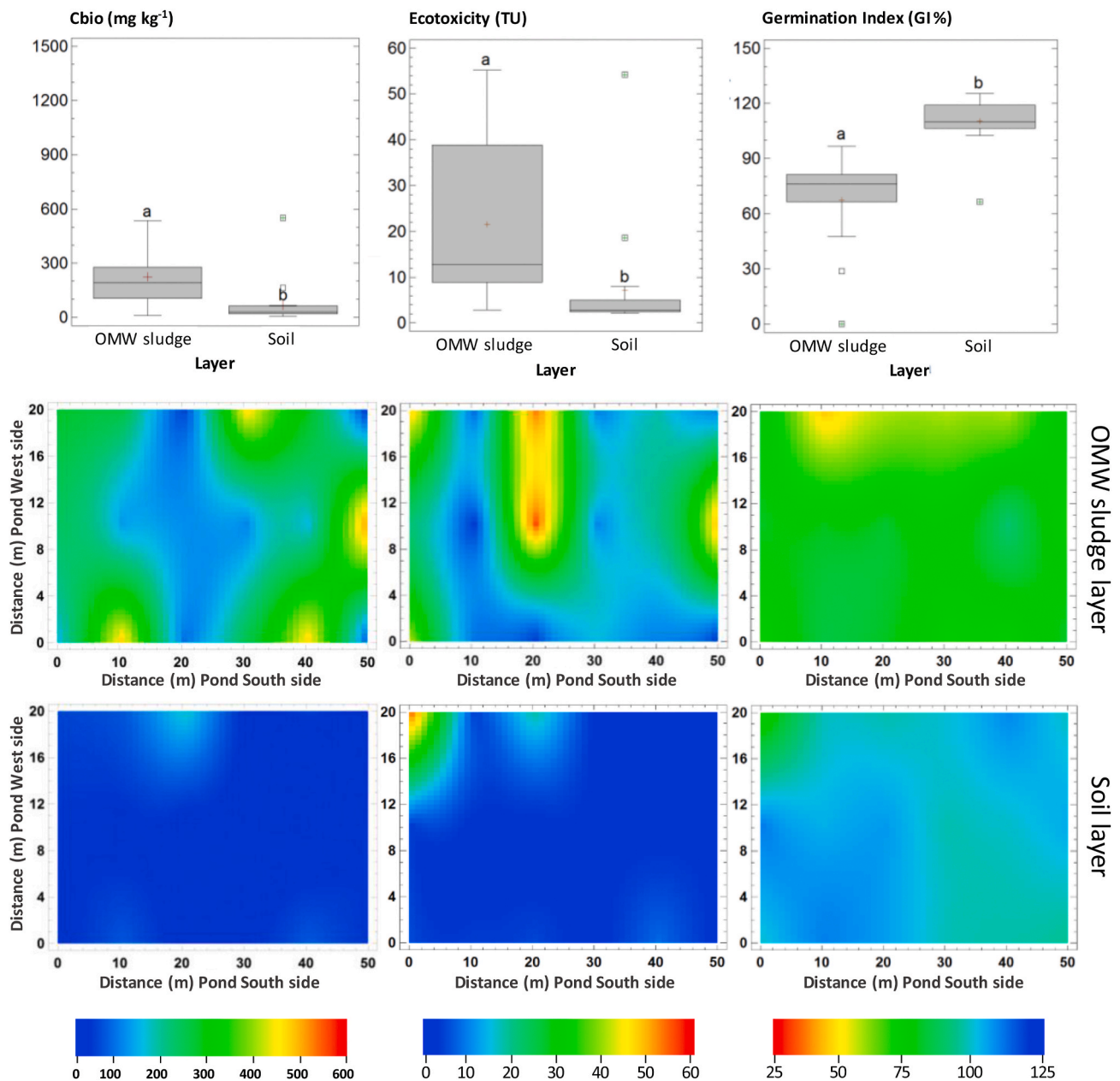
Fig. 6. Comparison and spatial distribution of Microbial biomass Carbon (Cbio), ecotoxicity (Toxicity Units-TU) and phytotoxicity (Germination Index-GI%) in OMW sludge (superficial) and soil (Deep) layers of pond 2. Results from 18 sampling points taken from each layer. Upper graphs show boxplots with average values within the box (+) (n = 18). The boxes bearing different letters were significantly different according to Fisher's LSD test (p < 0.05). Middle and bottom graphs show spatial distribution of the parameters in the two layers of the pond based on ordinary kriging method. The colour key at the bottom gives the value of the parameter represented in the maps. Graph and maps in each column refer to same parameter. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

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Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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during the field trials in OMW evaporation ponds.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jenvman.2020.111810.

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