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Treatment of olive mill wastewater using ozonation followed by an encapsulated acclimated biomass

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ABSTRACT

The environmental impacts caused by Olive Mill Wastewater (OMWW) are a concern for both developing and developed countries. In this study, an ozone pretreatment combined with a fixed biomass bio-treatment using the Small Bioreactor Platform (SBP) capsules technology encasing a pure culture of a phenol-degrading OMWW isolate named *Delftia* EROSY was implemented to reduce phenolic compounds and organic matter in OMWW.

Up to 90% of tannic acid (TA), a synthetic phenol model, was removed after the ozonation and biological stages. Ozone pretreatment of TA expedites the biological process by decreasing the time needed for the biodegradation of phenols.

Ozonation (ozone dose = 765 mg L^{-1} O_3) of OMWW demonstrated 20% COD and 61% total phenol removal, with an additional 36% increase in COD removal after the biological step (48 h). Interestingly, our results also showed that spectral absorbance can be used as a tool for monitoring ozonation followed by bio-treatment of OMWW. Absorbance results clearly demonstrate that bio-treatment is necessary for degrading not only phenolic compounds, but also phenol transformation products and the high organic load of the OMWW, following the ozonation step.

1. Introduction

Olive mill wastewaters (OMWW) are toxic industrial wastewaters due to the presence of toxic compounds and a high load of organic compounds [1]. OMWW is characterized by a dark reddish-black color, mildly acidic pH, high organic content and toxic materials which are composed mainly of sugars, tannins, pectins, polyphenols, polyalcohols and lipids. These compounds are persistent, and thus very difficult to treat by physical and chemical methods or biodegradation [2]. OMWW is characterized by high levels of chemical oxygen demand (COD) (in the range of 80–200 g L^{-1}), biochemical oxygen demand (BOD_5) (in the range of 50–100 g L^{-1}) and recalcitrant phenolic compounds (2–15 g L^{-1}), which are of the main cause for environmental problems arising from the discharge of OMWW. Even though the toxicity of this effluent is well-known, it is still discharged illegally into fresh water ecosystems or dumped on soils without proper treatment [3]. The scale of its environmental impact can be inferred from the fact that 1 m^3 of OMWW is equivalent to 100–200 m^3 of domestic sewage [4]. The negative effect of OMWW was demonstrated on soil microbial populations [1], on aquatic ecosystems, and even in air [5].

Treatment and disposal of OMWW is currently one of the most complicated environmental problems in the agro-industry [6]. The biotoxic properties of phenols in OMWW constitute a significant inhibitor of the biological processes that take place in common wastewater treatment plants (WWTPs). Municipal WWTPs do not present the desired performance with OMWW discharge. Treatment of OMWW together with municipal wastewater is thus not economically feasible, due to overload of the municipal wastewater that can induce an oxidative stress episode resulting in collapse of the bio-treatment process. Guidelines for managing OMWW through technologies that minimize their environmental impact and lead to a sustainable use of resources are therefore necessary, particularly in light of increasing olive oil

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production worldwide (2.6 million tons as published in the International Olive Oil Council 2016 newsletter [7]).

Pretreatment of OMWW should be designed to improve the wastewater quality and remove most of its toxicity. In addition to being technically feasible, OMWW treatment processes must be efficient, allow for easy and economical operation and consider the spatial distribution of olive oil production and the seasonality in harvest time. Various waste management practices have been reported in the last two decades, which apply physical, chemical and biological processes as well as their combinations due to the great variety of components found in the OMWW [8]. Some of those treatments include dilution, evaporation, sedimentation, filtration, coagulation-flocculation, adsorption on granular activated carbon, aerobic and anaerobic digestion, and treatments using fungi and bacteria. However, these technologies lead to limited biodegradability levels of organic matter and phenolic compounds [9]. The process efficiency, complexity and costs of installation, operation and energy may vary significantly. High cost is generally the main reason for not adopting efficient OMWW treatment methods [10]. Expensive treatment methods are not profitable, considering the short production period and the small size of most olive mills [11].

Interest in direct oxidation and advanced oxidation processes for the treatment of industrial effluents, as well as for the treatment of OMWW, has been growing in recent years [12–14]. Ozone (O_3) is a powerful oxidizing agent that selectively attacks compounds containing aromatic rings and double bonds. It is thus capable of causing oxidative degradation of many organic compounds such as polyphenols which are present in OMWW. Benitez et al. [15] observed ~20% COD reduction of OMWW for an initial COD of 10 g L^{-1} after 2 h of ozonation. This reduction was attributed to the oxidation and breakdown of larger organic compounds into smaller and less polluting ones [15]. COD reduction was ~20 and 60% when the ozone concentration (240 min ozonation time) increased from 22 to 60 mg L^{-1}, respectively [16]. However, ~80% phenol removal for the same ozonation time demonstrates the selectivity of ozone towards the toxic fraction of the OMWW (e.g., aromatic rings and double-bond compounds). Ozone pretreatment may thus be a viable procedure for subsequent bio-processes. Ozone is highly soluble in water, and therefore leaves no residuals. This leads to a safer environmental disposal profile.

Physicochemical systems combined with bio-treatments have been reported in the literature to reduce phenolic compounds and organic matter. Ozonation combined with bio-treatment can convert the non-biodegradable and hard-to-biodegrade compounds into readily biodegradable compounds for the bio-treatment, resulting in safer effluent disposal into the environment. For example, Benitez et al. [15] investigated degradation of organic matter by either ozonation or aerobic degradation, and by a combination of the two. Contaminant load, determined by COD and total aromatic and phenolic contents, exhibited 17–28% reduction from the initial COD after the ozonation stage, 76.2% in the aerobic degradation process (HRT = 7 days) and 82.5% in the combined ozonation and aerobic degradation stages. The higher COD reduction obtained in the combined process was associated with the removal of some inhibitory phenol compounds, resulting in decreased toxicity and increased biodegradability. Another study showed that ozone pretreatment followed by anaerobic digestion improved biodegradability, thus enhancing the removal of polyphenolic compounds [17], which is considered the best available technology according to the 96/61/EC directive.

However, microorganisms in a suspension growth state might present some sensitivity to the polyphenols and other ozonated organic transformation products in OMWW, due to direct interactions between the bacteria and toxicants. This is partially prevented in the encapsulated growth state, due to slower diffusion through the capsule's membrane into the encapsulated biomass. No study has, to date, demonstrated the feasibility of ozone pretreatment followed by an aerobic biological process with encapsulated microorganisms for treating OMWW. The goal of the present study was, therefore, to investigate the ozonation process coupled with the encapsulated biological process for oxidation of phenolic compounds and organic matter from a synthetic phenol model (tannic acid – TA) and on actual OMWW. The ozonation step was designed to enrich the wastewater with oxygen, reduce the toxicity of phenolic compounds and the organic load and increase OMWW biodegradability. Bioaugmentation of an encapsulated phenol-biodegrading bacterial isolate, previously isolated from OMWW, was then conducted and studied for its phenol and organics removal capabilities.

2. Materials and methods

2.1. OMWW characterization and sampling

The raw OMWW used in this study was collected during the oil harvesting season (November-December 2016) from a local council in the Central District of Israel named 'Zemer' that practices a three-phase extraction system (Fig. S1, S2). The collected wastewater was kept in sealed plastic containers at 4 °C with no exposure to light. Olive oil wastewater composition varies depending on the olives' properties and maturity (harvest time), storage time, cultivation soil, presence of pesticides and fertilizers, climatic conditions, the extraction process used and the operating conditions [11]. The raw OMWW and 1:10 diluted OMWW were characterized and the results are presented in Table 1. COD and TPh were analyzed since they are considered to be the major pollutants in the OMWW. Variability in the OMWW total phenols (TPh) concentration with time is presented in Fig. S3.

2.2. Chemical reagents and wastewater analysis

Tannic acid and Folin-Ciocalteu (F-C) phenol reagent were purchased from Sigma-Aldrich (Germany). TPh content was determined using the F-C method according to the protocol of [18]. In brief, 0.1 mL of a quenched sample was placed in a 1 mL tube and 0.2 mL of 10% (v v^{-1}) F-C phenol reagent in water were added. The samples were then incubated for 30 min at room temperature and 0.8 mL aqueous 700 mM sodium carbonate solution were added. The samples were incubated at room temperature for an additional 2 h, after which 0.2 mL were transferred into 96-well plates and absorbance was determined at 735 nm using a multimode microplate reader (Spark 10 M, Tecan, Männedorf, Switzerland). Chemical Oxygen Demand (COD) test kits with a measuring range from 0 to 15,000 mg L^{-1} O_2 were purchased from Lavibond (England), based on the dichromate method and determined in a Hach spectrophotometer. UV/Vis analyses were conducted with an Agilent Cary-100 spectrophotometer. Spectra were collected in quartz cuvettes using a wavelength range of 200–800 nm. Working solutions were prepared by dilution with deionized (DI) water according to the sample COD (Direct-Q3 UV System, Millipore).

2.3. Experimental setup

2.3.1. Ozonation experiments

Ozonation experiments were performed in a semi-continuous batch reactor, allowing continuous addition of ozone to a fixed batch of aqueous solution (Fig. 1). Ozone gas was generated from pure oxygen using an oxygen-fed ozone generator (up to 4 g h^{-1}; BMT 802 N,

Table 1
Characteristics of raw OMWW and with 1:10 dilution from a three-phase manufacturing process.

	pH	COD (mg L^{-1})	TPh (mg L^{-1})	EC (mS cm^{-1})
Raw OMWW	4.73 ± 0.12	13,160 ± 6.58	8,930 ± 0.8	11.39 ± 2.54
1:10 dilution	4.76 ± 0.06	10,430 ± 0.13	1,000 ± 1.2	6.43 ± 3.15

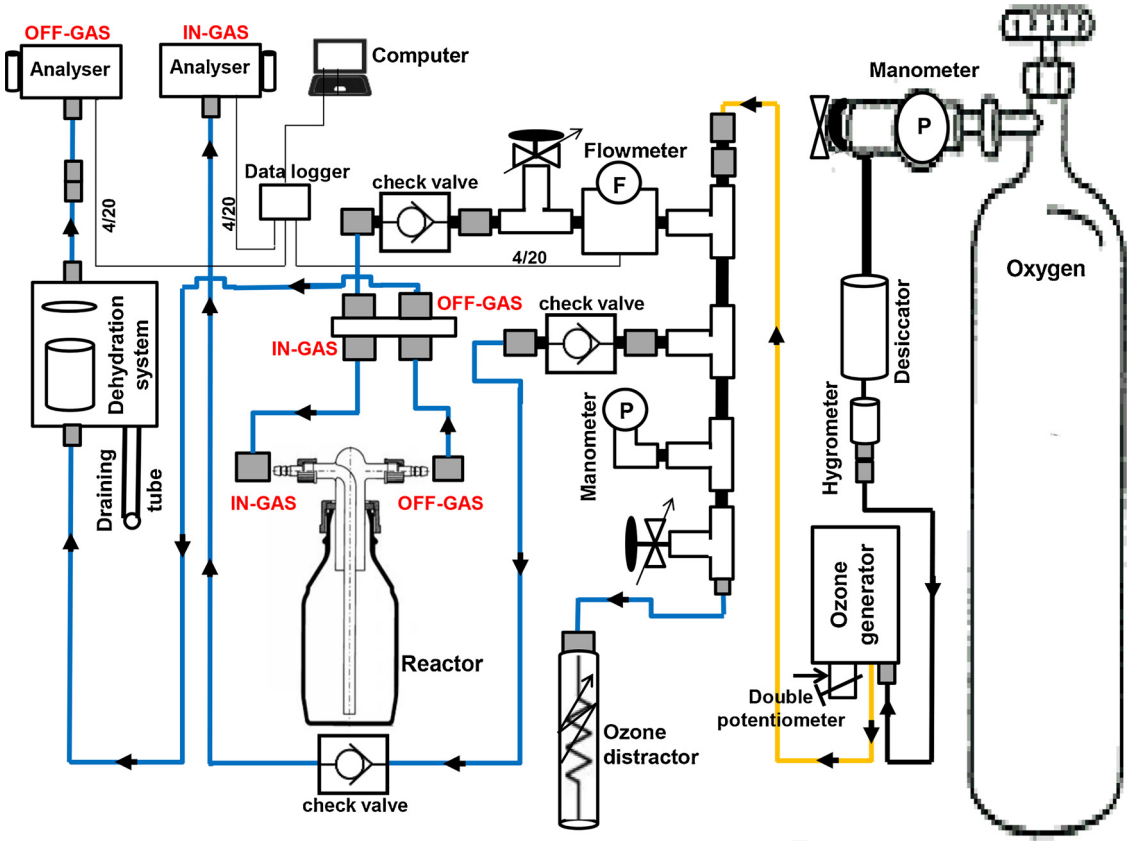

Fig. 1. A schematic drawing of the semi-batch ozonation reactor.

Germany). Ozonation experiments were conducted in a 1 L glass reactor (height: 20.5 cm, diameter: 10.8 cm) with a 12 mm diameter diffuser made from ceramic materials (nominal pore size 160–250 μm). The oxygen-ozone gas mixture was bubbled directly into the OMWW, with an average gas flow rate of 0.35 L min^{-1} at room temperature, allowing continuous addition of ozone to the OMWW under mixing by bubbling.

Ozone concentrations at the reactor input and output were continuously monitored via two sampling ports connected to UV ozone analyzers (BMT 964BT). Samples of the OMWW were taken for further analyses at the designated time points. The transferred ozone dose (TOD; accumulated amount of ozone transferred) was calculated using the formula (1):

$$\textit{Transferred Ozone Dose}\,(mgL^{-1}) = \frac{\sum (C_{o3,in} - C_{o3,out})_{mg\,L^{-1}} \times gas\ flow\ rate_{L\,min^{-1}} \times t_{min}}{Volume_L} \quad (1)$$

Where $C_{O3,\ in}$ = ozone concentration in the inlet gas, $C_{O3,out}$ = ozone concentration in the outlet gas, representing the unreacted ozone exiting the reactor. Volume $_L$ = water volume in the reactor. A schematic diagram of the experimental set-up is shown in Fig. 1.

2.3.2. Microorganism isolation, 16S rRNA-based phylogeny and sequence accession

Microorganisms originating from OMWW were isolated on MSM-agar plates supplemented with 50 mg L^{-1} phenol (Sigma, Israel) by their ability to degrade phenol as the sole carbon source as described in Kurzbaum et al. [19]. After incubation at 37 °C for 48 h, the fastest growing colonies were isolated using several isolation cycles. In order to verify the phylogenetic affiliation of the selected isolate, one colony was picked and resuspended directly into a PCR mixture for amplification of the 16S rRNA gene fragment (direct colony PCR). The reaction (25 μL) contained: 10 μL APEX 2XRedTaq Mix (Genesee Scientific, USA), 5 pmol of each primer [27 F (AGAGTTTGATCMTGGCTCAG) and 1513R (ACGGYTACCTTGTTACGACTT)], 12.5 μL DDW and 1 μL DNA as template. The PCR procedure was as follows: DNA was denatured at 95 °C for 5 min, followed by 30 cycles at 95 °C for 30 s each, 58 °C for 30 s and 72 °C for 1 min, followed by 5 min at 72 °C. The PCR product was sequenced from both ends by Hy-labs (Rehovot, Israel) and a consensus sequence was constructed.

The evolutionary history was inferred using the Minimum Evolution method [20]. The optimal tree with the sum of branch length = 0.26061275 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1050 replicates) are shown next to the branches [21]. The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Kimura 2-parameter method [22] and are in units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). The ME tree was searched using the Close-Neighbor-Interchange (CNI) algorithm [23] at a search level of 1. The Neighbor-joining algorithm [24] was used to generate the initial tree. All positions with less than 60% site coverage were eliminated. That is, less than 40% alignment gaps, missing data, and ambiguous bases were allowed at any position. The final dataset included 1380 positions. Evolutionary analyses were conducted in MEGA7 [25].

2.3.3. Encapsulation of the OMWW isolate in membrane-based capsules ('Small-Bioreactor Platform' (SBP) technology)

The technical details and the capabilities of the membrane-based capsules named Small-Bioreactor Platform (SBP) technology are described in detail in patent number PCT/IL2010/000256 [26] and in Menashe and Kurzbaum [27] and Azaizeh et al. [28]. Briefly, the SBP capsule contains a suspension of the selected pure culture which in this study was an OMWW isolate (closest to *Delfina tsuruhatensis*). The

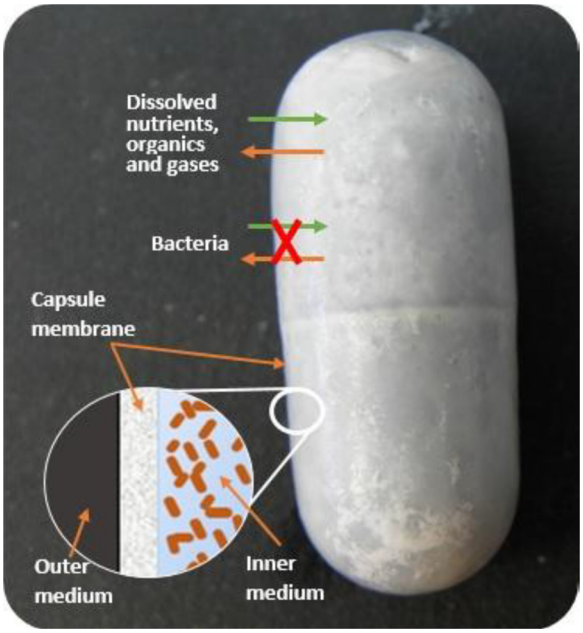

Fig. 2. A photograph combined with an illustration of the SBP capsule. The SBP capsule is 0.8 cm in diameter and 2.5 cm long, and is made of a cellulose acetate microfiltration membrane. The SBP capsule contains the OMWW isolate as a suspension inside a confined environment. The capsule membrane, which is permeable only to dissolved materials, does not allow trafficking of the microorganisms across the membrane. The capsule therefore holds the pure culture suspension for a long period of time (months) in a viable and uncontaminated state.

OMWW isolate was grown to achieve a high biomass concentration followed by a freeze-drying procedure and encapsulated within the SBP capsules as detailed in Kurzbaum et al. [29] (Fig. 2). Briefly, the SBP capsule is made of a cellulose acetate microfiltration membrane. Its size is 0.8 × 2.5 cm. The internal part of the capsule is hollow and the membrane is permeable only to dissolved nutrients and gases. It therefore does not allow trafficking of microorganisms across the membrane into and out of the capsule. The SBP capsule holds the supplemented culture suspension in a viable state for at least 2 months, preventing the biomass dilution while biodegrading pollutants in the wastewater.

2.3.4. SBP capsules activation and acclimation to the experimental medium

Since the SBP capsules are prepared with a bacterial culture that underwent a freeze- drying procedure, the encapsulated culture must be reactivated. This activation stage was performed in 500 mL Erlenmeyer flasks in sterile Minimal Salt Medium (MSM). The MSM medium was composed of (g L^{-1}): $NaHPO_4{\cdot}12H_2O$, 6.15; KH_2PO_4, 1.52; $(NH_4)_2SO_4$, 1; $MgSO_4{\cdot}7H_2O$, 0.2; $CaCl_2$, 0.038; and 10 mL of a trace element solution containing (g L^{-1}): EDTA, 0.5; $FeSO_4{\cdot}7H_2O$, 0.2; $ZnSO_4{\cdot}7\ H_2O$, 0.01; $MnCl_2{\cdot}4H_2O$, 0.003; H_3BO_3, 0.03; $CoCl_2{\cdot}6H_2O$, 0.02; $CuCl_2{\cdot}2H_2O$, 0.001; $NiCl_2{\cdot}6H_2O$, 0.002; $NaMoO_4{\cdot}2H_2O$, 0.003. The final pH of the medium was adjusted to 7 [30]. The MSM growth medium was prepared with distilled water and autoclaved at 121 °C for 20 min. The capsules were supplemented with fresh MSM every 48 h. The medium contained increasing TA concentrations (up to 150 mg L^{-1}) in the TA experiments and increasing OMWW percentages (1, 3, 10, 50 and 100%) in the OMWW experiments. Fig. S4 presents the SBP capsule activation and acclimation to the experimental medium.

2.4. Tannic acid degradation vs. ozonation times

A series of ozonation experiments were completed in a semi-batch mode using a 0.5 L flask filled with 250 mL of a solution of 150, 300, 1000 mg L^{-1} TA in DI water (pH = 3.75, 3.52, 3.7, respectively) in order to evaluate TA degradation as a function of different ozonation times. These concentrations were chosen based on TPh concentrations in OMWWs tested in preliminary experiments and according to the mg O3/mg TA ratio published in the study by Peretz et al. [39]. Samples were taken at various time points: 3, 5, 7, 10, 15, 20, 25, 30, 40, 45, 50, 55 and 60 min (TOD = 215, 297, 326, 364, 415, 489 mg O_3 L^{-1}, for 3–20 min, respectively) for total phenol analysis.

2.5. Tannic acid degradation at different ozonation times followed by bio-treatment using the SPB capsules

A stock of 150 mg L^{-1} TA in MSM solution was prepared and covered with aluminum foil to protect from light exposure. A volume of 500 mL of the solution was ozonated in a 1 L reactor for 1.5, 3 and 10 min (TOD = 144, 215, 283 mg O_3 L^{-1}) with an average gas flow rate of 0.35 L min^{-1}. Samples were taken before and after ozonation. Both solutions, before and after the ozonation process, were divided into six flasks with a volume of 250 mL, each filled with 150 mL solution. Triplicate samples were performed for TA before and after ozonation. Five SBP capsules were placed inside each of these flasks, and all were incubated for 48 h at 30 ± 1 °C under orbital shaking at 100 rpm. Test samples from each flask were taken every 12 h.

2.6. Experimental setup of OMWW degradation by ozone and the encapsulated Delftia EROSY culture

Semi-batch experiments were conducted using different TOD values (ozonation times) to examine the effect of ozonation on the subsequent bio-treatment with *Delftia* EROSY capsules. The ozone dose was estimated by continuous measurement of the ozone concentration in the gas phase at the inlet and outlet (off-gas) of the reactor. The accumulated TOD of 1000 mg L^{-1} OMWW (diluted 1:10) was plotted as a function of time (Fig. S5). A clear linear correlation was observed, suggesting a first-order reaction of the ozone with the organic matter.

A 1 L flask was loaded with 500 mL OMWW diluted 1:10 with DI water, while ozone was continuously fed into the reactor for 60 min. After the ozonation procedure, the pH was adjusted to 7–8.5 using 3 M ammonium nitrate (NH_4NO_3) and 5 M sodium hydroxide (NaOH). Since OMWW contains a high organic load which results in an anoxic medium, it was necessary to maintain extensive aeration during the experiments with the SBP capsules using the ozonation system which operated in a pure oxygen mode with a constant feed flow rate of 1 L min^{-1}. Under these conditions (48 h incubation time), 15 SBP *Delftia* EROSY capsules were introduced into the reactor containing the ozonated OMWW. Each experiment was conducted in triplicate up to 48 h, and samples were removed periodically for TPh and COD analysis, since these are considered the main contributors to the total pollutant load.

3. Results and discussion

3.1. Bacterial strain isolate

The bacterial strain from the OMWW was found to be aerobic, Gram negative, with a rod-shaped morphology and motile (data not shown). The primary sequence alignment carried out by NCBI nucleotide blast search revealed that the OMWW isolate belongs to the *Delftia* genus. A Maximum Likelihood method analysis gave the phylogenetic position of this isolate as closest to *Delftia tsuruhatensis* (Fig. 3). The nucleotide sequence was submitted to the GenBank at NCBI, and was assigned the accession number MF375113. For the current study, this isolate was named *Delftia* EROSY.

Bacteria of the genus *Delftia* are environmental microorganisms with a wide geographical distribution. They are versatile microbes with diverse metabolic capabilities and easily adapt to different environments [31]. As a microbial model of biodegradation studies, *Delftia* spp.

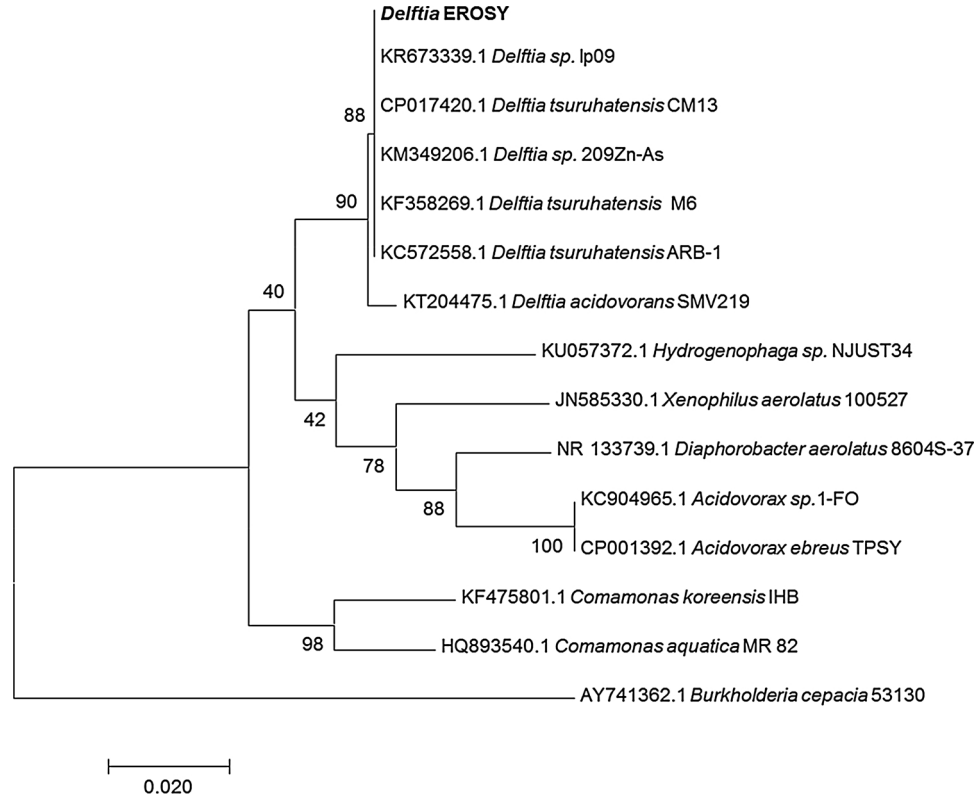

Fig. 3. Neighbor-joining phylogenetic tree based on 16S rRNA gene sequences obtained from *Delftia* EROSY (1380 positions) and related strains. *Burkholderia cepacia* was used as an outgroup. Bootstrap values based on 1050 re-samplings are shown at branch nodes.

strains were widely used in bioremediation and bioconversion of contaminants, mainly heavy metal (lead, chromium, etc.) and antibiotics [32,33], nitrification and aerobic denitrification [34]. Interestingly, only few studies reported their ability to degrade phenolic compounds [35,36]. This is therefore the first time that *Delftia* sp. showed successful degradation of tannic acid and other phenols present in OMWW up to a concentration of 1000 mg L^{-1} (Subsections 3.3 and 3.4).

3.2. Tannic acid degradation vs. ozonation times

Ozonation was examined on 150, 300, 1000 mg L^{-1} TA solutions (measured as total phenol) to simulate conversion of OMWW. As expected, a decrease in TA concentration was obtained with increased ozonation times (or TOD) (Fig. 4). For example, removal of 40% of 150 mg L^{-1} TA was obtained within the first 3 min of ozone reaction time, 60% within 5 min, and a tailing was observed above 7 min that

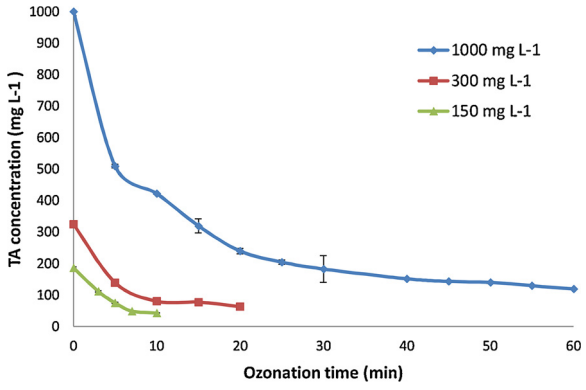

Fig. 4. Effect of ozonation time on removal of tannic acid at initial concentrations of 150, 300, 1000 mg L^{-1}. TOD = 310, 375, 436 mg L^{-1} respectively. Gas flow rate: 0.35 L min^{-1}. Experiments were conducted in triplicate.

corresponds to removal of 74% and 77% at 7 and 10 min ozonation time, respectively. The TA concentration decreased continuously with ozonation, up to above 7 min where removal rate decreased. These results are consistent with other studies that showed a rapid initial first order reaction rate constant for ozonation of phenols [37–39]. Furthermore, the decrease in TA concentration can correspond to first order kinetics as discussed extensively in Peretz et al. [39] who observed a three-phase TA decomposition kinetics at a very high TA concentration of 60,000 mg L^{-1}. In addition, the initial pH of 3.69 before ozonation decreased to 3.02 within 10 min of the ozonation process, due to formation of intermediate acids such as weak organic or phenolic acids. In this study, we aimed to use conditions similar to actual OMWW which is acidic in nature. pH values can have a crucial effect on the ozonation process, with acidic pH favoring direct (and selective) ozone oxidation and basic pH favoring non-selective hydroxyl radical (·OH) reactions [40]. Ozone consumption by TA involves a mechanism of ozone attack on the aromatic rings leading to oxidation of the polyphenol chain. At low pH, the intermediate byproduct from the phenol oxidation process is usually, but not limited to, a quinone. Quinone formation can activate a process of polymerization until a termination point of free radical deficiency is reached. Therefore, although ozone has excellent chemical oxidation characteristics and is able to destroy toxic organic compounds such as phenols, toxic intermediate compounds (to pure bacterial cultures and mixed microbial cultures) can still be generated during the early stages of the ozonation process [41–43]. This formation of intermediate compounds following ozonation may further affect the bio-treatment performance by the SBP capsules, as will be further discussed. Nevertheless, Ji-min et al. [44] showed the impact of ozonation on the observed color of the solution due to decomposition of TA, which was not observed in the current study, probably due to the relatively low TA concentration.

Table 2
A summary of the effect of the combined ozone pretreatment at different ozone doses (ozonation times) followed by bio-treatment on the TPh concentration (%) removal. After the ozone treatment, all samples were incubated for 48 h (30 °C, 100 rpm). Each experiment was conducted in triplicate.

		Biological stage				
Ozonation time	0 h	12 h	24 h	36 h	48 h	TOD (mg L^{-1} O_3)
1.5 min	37 %	41 %	86 %	87 %	88 %	144
3 min	37 %	51 %	71 %	83 %	83 %	215
10 min	87 %	88 %	90 %	N. A	90 %	283

3.3. Tannic acid degradation after different ozonation times followed by bio-treatment with the SPB capsules

A successive combined process of different ozonation times (1.5, 3, 10 min) followed by the biological stage using SBP capsules was performed to evaluate the effect of the ozonation step on TA biodegradability. As expected, increasing ozonation time was expressed in an immediate and rapid reduction in the TA concentration (Table 2). 10 min of ozone treatment yielded 87% TA degradation, whereas 1.5 and 3 min of ozonation showed only 37% (TOD = 283, 144, 215 mg L^{-1} O_3, respectively). For both the 1.5 and the 3 min experiments, a major degradation of 86% and 71%, respectively, was obtained after 24 h.

According to the presented results, 10 min of ozonation results in the highest TA degradation rate, with further biodegradation of up to 90%. A 1.5 and 3 min ozonation resulted in lower removal rates, and the biological stage was then responsible for the remaining TA removal of up to 88% and 83%, respectively. Consequently, 1.5 min of ozone pretreatment might theoretically be sufficient for 24 h bio-treatment of TA and is similar to 10 min of applied ozone without bio-treatment. However, with OMWW, the additional bio-treatment is necessary for degrading phenol transformation products and the high organic load of the OMWW following ozone pretreatment. The impact of the biological stage ceases to show additional TPh removal after 12 h for the 10 min ozone pretreatment, while a moderate and almost steady decline of the COD and TPh concentrations was observed after ozonation for 1.5 and 3 min. It can therefore be concluded that ozone pretreatment of TA expedites the biological process by decreasing the duration of the biological process which is necessary for achieving the same degradation rate.

3.4. Tannic acid 'control' experiments

Table 3 presents the TA degradation of three different control experiments: (1) only ozonation (10 min, TOD = 310 mg L^{-1}), (2) only the biological stage with the SBP capsules, and (3) no treatment, all incubated for 48 h (30 °C, 100 rpm). A reduction in the TA concentration was observed in both treatment systems: ozonation and biological (SBP capsules) experiments, until stability in the TA concentration was achieved for the biological stage (36–48 h). After the ozone pretreatment, the TA concentration was reduced to 27% within minutes (data not shown). Ozonation and capsules with 12 h of bio-treatment resulted in a TA degradation of 86%, which is similar to the capsules without ozonation (88%). However, this was achieved at the expense of 24 h of incubation. As expected, there was no significant change in the control test with no treatment (no ozonation and no bio-treatment).

Table 3
TA concentration reduction as a function of time (h) in three different experiments (only ozonation, only capsules, and no treatment). Samples were taken at 12 h intervals. Each experiment was conducted in triplicate.

	Ozonation/ capsules	0 h	12 h	24 h	36 h	48 h
Control 1	Only ozonation	**27 %**	**86 %**	87 %	N.A	N.A
Control 2	Only capsules	–	46 %	**88 %**	97 %	98 %
Control 3	No treatment	–	0 %	11 %	13 %	16 %

* 1 h ozonation (TOD = 789 mg O_3 L^{-1}).

Table 4
COD and TPh concentrations and % removal with or without 1 h of ozonation (TOD = 765 mg L^{-1} O_3) of diluted OMWW (1:10) followed by bio-treatment for 48 h. Each experiment was conducted in triplicate.

Treatment	COD, mg L^{-1} or COD removal (%)	TPh, mg L^{-1} or TPh removal (%)
Values at the beginning	10,433	1,000
1 h O_3	20%	61%
1 h O_3 + 48 h bio-treatment	36%	61%

* TOD = 765 mg O_3 L^{-1}.

3.5. OMWW degradation with ozone pretreatment followed by the biological SBP capsules process

It is expected that ozone pretreatment will oxidize the phenols in the raw OMWW. For example, Karageorgos et al. [16] identified compounds present in raw OMWW including polyphenols, carboxylic acids, alcohols alkanes, alkenes and esters. This study demonstrated that ozonation of the raw OMWW was able to remove part of the phenolic content, yielding less toxic byproducts (carboxylic acids and alcohols). Ozone also increases the mixed liquor dissolved oxygen level (14% of ozone on a mass basis in an ozone/oxygen mixture), which is especially important with high organic loads for biological aerobic treatment.

The OMWW TPh concentration (C/C_0) vs. ozonation time is presented in Fig. S6. Nevertheless, the main goal of this experiment was to demonstrate the combined ozonation-capsules biodegradation of OMWW (diluted 1:10 with DI water). Table 4 presents the COD and TPh concentrations before and after 1 h of OMWW ozonation (TOD = 765 mg L^{-1}), followed by bio-treatment for 48 h. Exposure of OMWW to 1 h of ozonation resulted in a 17–21% reduction of COD and 59–62% reduction of TPh (Table 4). Bio-treatment after ozonation presented an additional reduction of COD in the range of 34–37%, without additional phenol reduction (Table 4). In the study of Azaizeh et al. [28], the phenolic compounds fraction (total phenol/COD; g g^{-1}) was 16% of the total COD removed. It is therefore suggested that in our study, the ozone pretreatment targeted mainly the phenolic fraction and reduced its content in the OMWW. This may explain why no additional phenol reduction was observed after the bio-treatment.

Phenols are a cytotoxic element and oxygen scavengers. Reduction of this fraction in a pretreatment is therefore critical for the subsequent bio-treatment. In this study, phenol reduction during the ozone pretreatment did not influence the bio-treatment, possibly due to phenol fractions variation within the OMWW. Phenols have two major fractions within the OMWW: (1) dissolved phenol and polyphenols which present a polymer structure; (2) polyphenols which are less dissolved and tend to settle or form a colloidal suspension of particles and are therefore less available to the bacteria in the mixed liquor [11,45]. Thus, ozone treatment may target the polyphenol fraction (in addition to the free phenol molecules), resulting in a negligible effect on the bio-treatment, as observed in the current study.

Since the bio-treatment in this study contained a bacterial strain that biodegrades phenols (encapsulated *Delftia* EROSY), it is not clear why a significant phenol reduction was not observed during this treatment. This may be explained by two possible reasons: the presence of other substrates within the OMWW that require less energy investment for biodegradation compared to phenol [46] and the oxidative conditions within the mixed liquor. When phenol is used as a carbon source, the bacteria must cleave the benzene ring in order to exploit it as an energy source. The benzene ring is a stable structure which

necessitates expenditure of energy by the bacteria in order to break it down and use it as a carbon source. Only few bacterial strains have the ability and the metabolic paths necessary for using phenol as a carbon source. The oxidative conditions during bio-treatment of OMWW usually exhibit insufficient dissolved oxygen for further oxidation of phenolic compounds by the microorganisms. This may reduce the microorganisms' ability to biodegrade phenols as a carbon source in high organic mixture loads, such as those found in OMWW. Another explanation (mentioned above), is the formation of inhibiting byproducts resulting from ozonation. Even though ozone removes refractory phenolic compounds, thus improving biodegradability by decomposing the polyphenolic chain into smaller molecules, it may also generate numerous intermediates which may be compounds that disrupt the bacterial population within the mixed liquor (despite being less toxic than the original phenols).

The transferred ozone dose (TOD) (Eq. (1), as previously defined, is the accumulated amount of ozone transferred to the water that reacts with the examined organic compounds. This is an empirical design parameter that is usually determined for ozonation of trace organic compounds in drinking water and wastewater effluents [47,48] or in pollutants such as dyes and textiles in industrial WW [49]. Nevertheless, this parameter is rarely determined in ozonation of OMWW (Table 5).

Table 5 shows that a single ozonation process did not remove more than 10% of the Table 5 shows that a single ozonation process did not remove more than 10% of the COD [50], whereas 50% removal of TPh was observed following the same ozonation time (e.g., 5 h). In this context, ozonation time and maintenance costs must be evaluated when scaling up, since most of the presented studies implemented ozone for 3 h. In this study, ozonation was conducted only for 1 h, which resulted in a similar COD removal of 20% in the combined treatments of UV/UF followed by O3 [52,57]. Nevertheless, the biological step in this study increased the COD removal up to 36%, and thus improved the efficiency of the treatment by reducing the organic load. Interestingly, 5 h of ozonation led to less than a 10% decrease in the total COD, due to the selectivity of ozone towards double bonds, without reducing other components such as carbohydrates [50]. Moreover, the initial COD in the current study was significantly higher compared with other studies (Table 5), resulting in a greater difficulty in removing the OMWW organic matter.

The required amount of SBP capsules per cubic meter of wastewater is mainly dependent on the organic matter concentration, the phenol compounds concentration and the hydraulic retention time of the process. The following parameters were taken into account for the current aforementioned treatment model that combined ozone treatment as a pretreatment followed by bio-treatment, in order to estimate the cost of the SBP treatment: Initial COD concentration in a range of 10–20 g/L, phenol concentration less than 1 g/L and hydraulic retention time above 48 h (bioreactor volume of at least 20 cubic meters). The estimated amount of SBP capsules required for this process is 5000 units, since it is presumed that 250 SBP capsules are required for each cubic meter of treated OMWW for hydraulic retention time of 48 h. The use of a single dose of 5000 SBP capsules is estimated to be able to treat approximately 600 cubic meters of OMWW (each SBP capsule is active for a period of 2 months) and the commercial data obtained from BioCastle Water Technologies Ltd. suggest that the cost of each cubic meter of treated OMWW is 5.41 USD for the encapsulated biomass provided in the SPB capsules.

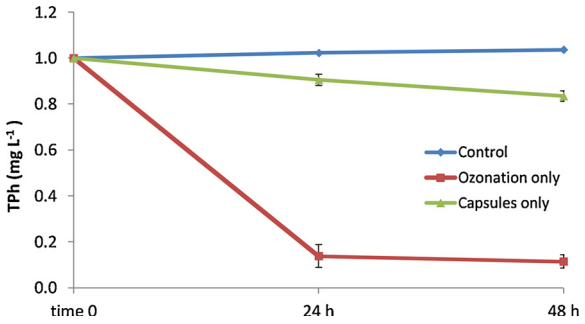

Fig. 5. Three different experiments with an overall incubation time of 48 h; 'Control' is without any treatment, 'Ozone only' is with 60 min ozonation but no addition of capsules, 'Capsules only' is with SPB capsules but no ozonation. Concentrations were normalized to values between 0–1.

3.6. OMWW 'control' experiments

A series of control trials with OMWW with an overall incubation time of 48 h were performed (Fig. 5). The initial OMWW concentration measured as total phenol was different in each experiment, due to the variability in parameters of the wastewater over time, despite its refrigerated storage. The concentrations were therefore normalized to 0–1. The three experiments were performed as follows: 'ozonation only' for 1 h with no addition of capsules after ozonation ('ozonation only'), only capsules without ozone pretreatment ('capsules only') and the OMWW with no treatment ('control').

As predicted, the control test showed no significant change during the incubation time. The 'capsules only' test showed 17% removal of TPh after 48 h. TPh removal immediately after ozonation reached 62% (data not shown). This result is similar to the combined ozonation-capsules OMWW experiment (see Table 4), and increased to 80% after 24 h of incubation.

In both tests, a minimal and insignificant decrease in TPh was observed in the second half of the experiments (between 24–48 h of incubation). These results raise the question of whether we can address pretreatment and reduction of the biological hydraulic residence time as a synergistic phenomenon. Ozone implementation with a retention time of 24–48 h could be sufficient for the organic compounds to biodegrade.

Table 5
Previous ozonation-based experiments on three-phase OMWW.

Reference	Type of treatment	Gas flow rate	Ozonation time	TOD	Initial COD	COD removal (%)	Initial TPh	TPh removal (%)
[9]	Ozonation + aerobic degradation	N.A	3 h	N.A	6000 ± 95 mg L^{-1}	76	180 ± 21 mg L^{-1}	82
[57]	Centrifugation + O_3	36 m^3 h^{-1}	2 h	N.A	134.1 g L^{-1}	31.5	9.1 g L^{-1}	63.6
[58]	1. UV + O_3	35 L h^{-1}	3 h	N.A	117.1 g L^{-1}	29	2.71 g L^{-1}	N.A
	2. Aerobic biodegradation + O_3	35 L h^{-1}	3 h	N.A	20 g L^{-1}	80	N.A	N.A
	3. O_3 + Aerobic biodegradation	35 L h^{-1}	3 h	N.A	20 g L^{-1}	87	N.A	N.A
	4. Aerobic biodegradation + UV/ O_3	35 L h^{-1}	3 h	N.A	45 g L^{-1}	81.8	N.A	N.A
	5. UV/ O_3 + aerobic biodegradation	35 L h^{-1}	3 h	N.A	45 g L^{-1}	90.7	N.A	N.A
[52]	UF + O_3	100 mL min^{-1}	N.A	N.A	2575 mg L^{-1}	20	234 mg L^{-1}	93
[50]	Ozonation	21.5 L h^{-1}	5 h	N.A	N.A	< 10	N.A	50

N.A, No available data.

3.7. Effect of pH on ozonation

The pH level is one of the most important parameters during ozonation, since it determines the ozone oxidation route. The preferred mechanism under acidic conditions is direct attack by molecular ozone (O_3), compared to hydroxyl radicals formation at higher pH levels [16,51,52]. The OMWW initial pH was 4.85, and the aim of the study was to work with actual OMWW, thus no pH alterations were done in order to study the effect of pH on OMWW ozonation efficiency. Phenolic compound removal was higher compared with COD removal (61% and 20%, respectively) after implementing the ozonation stage, probably due to the selectivity of ozone to organic compounds containing double bonds and functional groups as found in aromatic rings and phenols at lower pH [53]. Moreover, additional lowering of the pH after 60 min ozonation (final pH of 3.87) could lead to the formation of carboxylic acids and end products which are refractory to further oxidation by ozone, explaining the small reduction in COD levels. In this context, a slower kinetic rate might be useful for controlling and maintaining the reaction which is preferable towards phenol degradation only.

3.8. UV/Vis absorbance spectrum of OMWW

UV/Vis is a tool which can be used for detecting aromatic compounds, including polyphenols and phenols, and determining their degradation. Since OMWW contains high concentrations of aromatic compounds (e.g., polyphenols), UV/Vis spectra absorbance can be a useful method for detecting the elimination of phenols after conducting the combined treatment (as explained above). Four trend lines are presented in Fig. 6: three of the four trend lines refer to the combined process showing the original raw OMWW, the effluent after 1 h ozonation and after the 48 h biological process. The control trend line refers to the OMWW with the addition of SBP capsules without pretreatment, in order to evaluate the ozone pretreatment stage on the SBP biodegradation activity.

The UV/Vis spectra revealed two absorption peaks at 274 nm and 214 nm for raw OMWW (diluted 1:10). These two wavelengths are proportional to aromatic compounds content (Fig. 6). Both peaks showed a decline after the application of each of the treatments (1 h ozonation, single capsules biodegradation, combined ozonation-capsules), with a significant declining trend of the combined ozonation followed by the biological process. These results are attributed to the opening and decomposing of the aromatic rings into smaller less aromatic molecules, and even in a decrease in double bond molecules that characterize these sorts of substances. A decrease in the peak height of aromatic compounds in the UV/Vis spectra after ozonation is a well-known phenomenon due to the oxidative properties of ozone which destroys phenolic compounds, as reported previously by others [54].

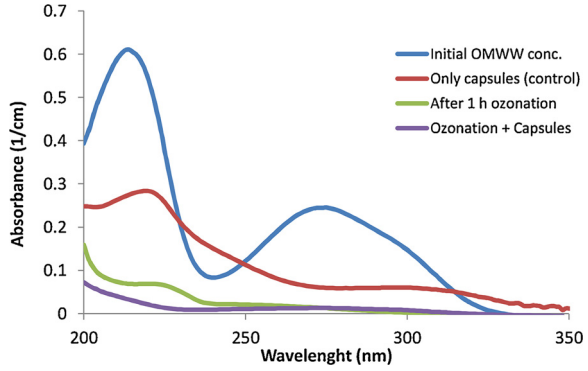

Fig. 6. UV/Vis spectrum of diluted OMWW 1:10: before and after 1 h ozonation; ozonation followed by bio-treatment with SBP capsules for 48 h; treatment with capsules only (no ozonation) incubated for 48 h.

The absorbance data of the combined process clearly demonstrate that ozonation of OMWW, followed by a bio-treatment, is necessary for degrading not only phenolic compounds, but also phenol transformation products and the high organic load of the OMWW following ozone treatment. Absorbance data are therefore an important tool for process monitoring of ozonation followed by bio-treatment of OMWW.

3.9. Delftia EROSY capsule activity under oxidative stress conditions

Aerobic respiration by microorganisms involves molecular oxygen (O_2) or oxidation of nutrients, resulting in the formation of numerous compounds that react with the biological target and are considered to be harmful to living organisms [55]. DNA, RNA, proteins and lipids are examples of these biological targets that are extremely sensitive to reactive oxygen species (ROS), generating reactive byproducts that result in damage to living organism cells [56]. Aerobic bio-treatment with the OMWW requires a continuous aeration system for the bacterial biodegradation activity. The effect of the pure oxygen in a flowing system on the viable bacterial counts was therefore examined. A fixed encapsulated *Delftia* EROSY biomass was used in the experiment. The experiments were conducted as follows: (a) ozone pretreatment of OMWW followed by addition of capsules to OMWW for 48 h incubation, (b) control experiments without the ozonation step and with addition of capsules to OMWW for 48 h incubation. Two samples were taken for determining the viable bacterial count: one after the capsules adaptation step (the beginning of the experiment) and another at the end point of the experiment, after 48 h of incubation. Three capsules were examined in each sample for determination of viable bacterial counts. An increase in viable counts was found after ozonation and 48 h of incubation ($4.38 \times 10^8 \pm 4.01 \times 10^7$ CFU mL^{-1}), compared to viable counts after the adaptation stage ($6.28 \times 10^7 \pm 7.07 \times 10^5$ CFU mL^{-1}). This increase may be attributed to the breakdown of the recalcitrant phenolic compounds which are toxic to living organisms, as mentioned previously in the article. Thus, their elimination after the ozonation step improves treatment efficiency for later biological processes [9]. Moreover, the viable counts after the ozone pretreatment and 48 h of the bio-treatment were higher than in the control test (no ozonation) ($5.69 \times 10^7 \pm 4.95 \times 10^6$ CFU mL^{-1}).

4. Conclusions

- An encapsulated fixed biomass was implemented after ozonation in order to treat OMWW and reduce its total phenol and COD concentrations.
- TA was used as a model substance that enables gaining insights into the obstinate OMWW.
- Pre-ozonation of TA reduced the subsequent biodegradation time.
- An encapsulated *Delftia* EROSY biomass degraded up to 1000 mg/L TA.
- *Delftia* spp. may serve as a good choice for future bioaugmentation studies in the remediation of industrial wastewaters containing phenolic pollutants.
- Combined ozone-encapsulated biomass treatment led to a 36% decrease in COD in the OMWW. This decrease was substantially greater than the decrease in TPh.
- Spectral absorbance can monitor pre-ozonation and biodegradation of OMWW.
- Absorbance data of the OMWW revealed the importance of the ozone pretreatment on degrading not only phenolic compounds, but also phenol transformation products.
- Post-treatment viable counts of the bacteria in the capsules demonstrated the capsules' superior resistance to ozone exposure and oxidative stress conditions.

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Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jece.2018.07.003.

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