

Nature-based solution to treat olive oil mill wastewater

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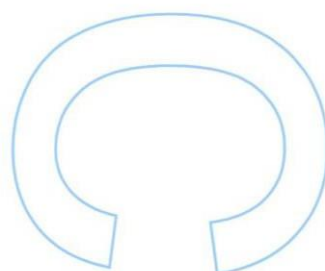
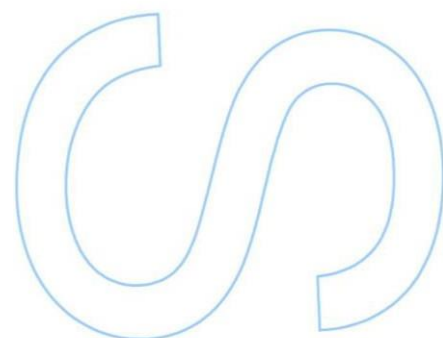
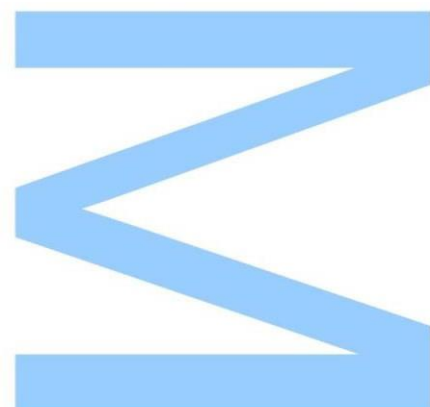
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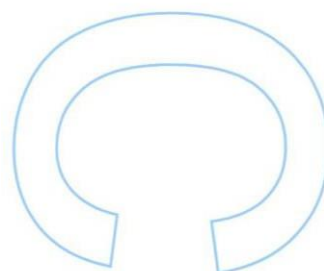
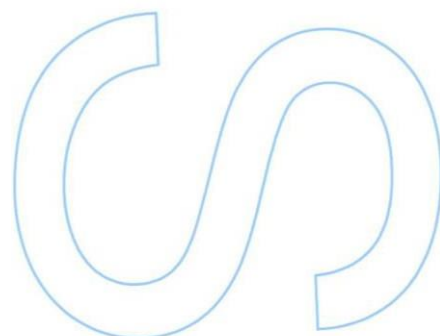
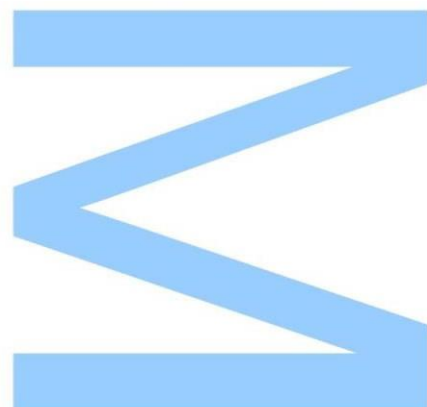




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Abstract

World olive oil production in 2020/21 exceeded 3 million tonnes and as the production continues to increase, also the environmental problems associated with olive oil waste grow. The extraction of olive oil lead to the production of olive oil mill wastewaters (OOMW), which is estimated to be 0.5-1.5 m³ per 1000 kg of olives. OOMW are characterised by their dark colour, acidic pH, strong odour, high organic matter content and complex and recalcitrant substances, in addition to phenolic compounds, being highly toxic to the biota. Another problem associated with these effluents, along with the seasonality of production (2-4 months) with high production rates, is the fact that most of these companies are small family businesses without the capital to invest in treatment facilities. Thus, in several countries, OOMW is often discharged untreated into soil or water bodies, leading to soil and water pollution, or disposal and storage in sedimentation ponds. Constructed wetlands (CW) are green treatment technologies, low cost and easy to maintain, which can be used to treat various types of effluents by adsorbing and mineralizing the compounds of the effluent when it passes through the substrate and plant roots.

Thus, in this work a lab-scale CW was developed, composed of a substrate of sand, LECA® and gravel, and *Iris pseudacorus* plants, with the aim of evaluating the efficiency of this technology in the treatment of OOMW, on the physicochemical properties and reduction of toxicity to aquatic organisms. In addition, considering the seasonality of OOMW production and in a circular economy perspective, the safety of using the CW substrate as soil amendment was assessed, through ecotoxicological tests with soil organisms.

The results showed an increase in the quality of the effluent, as well as a reduction in its toxicity. Furthermore, it could be concluded that the use of substrates from CW for soil amendment seems to be an ecologically feasible application, as it did not pose a danger to the tested organisms.

Keywords: Olive oil production, Constructed Wetlands, Ecotoxicological assays, Substrate valorisation

Resumo

A produção mundial de azeite em 2020/21 excedeu os 3 milhões de toneladas e à medida que a produção continua a aumentar, também o problema ambiental associado aos resíduos do azeite aumenta. A extração do azeite leva à produção de efluentes de lagar de azeite (águas ruças), cuja produção é estimada que seja 0.5-1.5 m³ por cada 1000 kg de azeitona. As águas ruças são caracterizadas por terem uma tonalidade escura, pH ácido, cheiro forte, elevado teor de matéria orgânica e substâncias complexas e recalcitrantes, para além de compostos fenólicos, sendo altamente tóxico para a biota. Outro problema associado a estes efluentes, além da sazonalidade (2-4 meses) com elevadas taxas de produção, é o facto de a maior parte destas empresas serem pequenos negócios familiares sem capital para investir em meios de tratamento. Desta forma, em muitos países, este efluente é muitas vezes descartado sem tratamento para o solo ou corpos de água, levando à poluição e contaminação do solo e da água, ou depositado e armazenado em tanques de sedimentação. A poluição da água por estes efluentes pode causar problemas ambientais graves, como o desenvolvimento de filmes de gordura na superfície, a depleção de oxigénio e eutroficação, conduzindo ao aumento da toxicidade. As fito-ETAR são tecnologias verdes de tratamento, de baixo custo e fácil manutenção, que podem ser usadas no tratamento de vários tipos de efluentes através da adsorção e mineralização dos compostos do efluente quando este passa pelo substrato e pelas raízes das plantas.

Assim, neste trabalho foram desenvolvidas fito-ETAR à escala laboratorial compostas por um substrato de areia, LECA® e gravilha, e plantas *Iris pseudacorus*, com o objetivo de avaliar a eficiência desta tecnologia no tratamento de efluente de lagar de azeite (EFLA), nas propriedades físico-químicas e redução da toxicidade para os organismos aquáticos. Para além disso, considerando a sazonalidade da produção de EFLA e numa perspetiva de economia circular, foi avaliada a segurança do uso do substrato das fito-ETAR no solo, através de ensaios ecotoxicológicos com organismos do solo.

Os resultados mostraram um aumento na qualidade do efluente, assim como a redução da sua toxicidade. Para além disso, foi possível concluir que o uso dos substratos das fito-ETAR para a correção do solo parece ser uma aplicação ecologicamente viável, uma vez que não representou perigo para os organismos testados.

Palavras-chave: Produção de azeite, fito-ETAR, ensaios ecotoxicológicos, valorização do substrato

Table of contents

Agradecimientos	2
Abstract	3
Resumo	4
List of tables.....	7
List of figures	8
List of abbreviations	9
1. Introduction.....	11
1.1. OLIVE OIL PRODUCTION.....	11
1.2. CONSTRUCTED WETLANDS.....	15
1.3. DISSERTATION OBJECTIVE AND STRUCTURE	16
2. Material and methods.....	18
2.1. PILOT CONSTRUCTED WETLAND.....	18
2.1.1. Experimental design	19
2.1.2. Physicochemical parameters	19
2.1.3. Ecotoxicological assays.....	20
2.2. SOIL AMENDMENT WITH CW SUBSTRATE – SAFETY EVALUATION	21
2.2.1. Soil preparation and physicochemical properties	21
2.2.2. Ecotoxicological assays.....	22
2.3. STATISTICAL ANALYSIS.....	25
3. Results and discussion	26
3.1. PILOT CONSTRUCTED WETLAND.....	26
3.1.1. Physicochemical parameters	26
3.1.2. Ecotoxicological assays.....	31
3.1.3. Overall assessment of the system's efficiency	34
3.2. SOIL AMENDMENT WITH CW SUBSTRATE – RISK ASSESSMENT	35
3.2.1. Physicochemical parameters	35
3.2.2. Ecotoxicological assays.....	36
4. Conclusion.....	42
5. References	43
6. Annexes.....	48

List of tables

Table I – Olive oil production per campaign from 2016 to 2021.....	11
Table II – Main composition of the olive oil mill wastewater (OOMW).....	13
Table III – Physicochemical characterization of soil samples.....	36
Table IV – EC ₅₀ values recorded after 5, 15 and 30 minutes of exposure of the bacteria <i>A. fischeri</i> to the samples of soil amended with the different substrates.....	36
Table 1A – Hoagland’s solution composition.....	48
Table 2A – ASTM hard water solution composition.....	48
Table 3A – Woods Hole MBL solution composition.....	49
Table 4A – Two-way and one-way ANOVA’s summary (df, F ratio and p value) and post-hoc Tukey test results, regarding physicochemical parameters of the E.75% and E.100%.....	49

List of figures

Figure 1 – Schematic representation of the composition of the constructed wetland (CW) units and lab scale vertical flow constructed wetland (VFCW).....	18
Figure 2 – Average values $\pm$ standard deviation of physicochemical parameters of E.CTL.....	26
Figure 3 – Average values $\pm$ standard deviation of volume retention of E.CTL, E.75% and E.100%.....	27
Figure 4 – Average values $\pm$ standard deviation of pH.....	28
Figure 5 – Average values $\pm$ standard deviation of electrical conductivity (EC).....	29
Figure 6 – Average values $\pm$ standard deviation of total nitrogen (Nt).....	30
Figure 7 – Average values $\pm$ standard deviation of chemical oxygen demand (COD) and total phosphorus (Pt).....	30
Figure 8 – Average values $\pm$ standard deviation of the colour.....	31
Figure 9 – Average values $\pm$ standard deviation of <i>R. subcapitata</i> growth rate exposed to several dilutions of raw OOMW (E.100%).....	32
Figure 10 – Average values $\pm$ standard deviation of <i>R. subcapitata</i> growth rate exposed to several dilutions of samples after 48h (A), 72h (B) and one week (C).....	33
Figure 11 – Average values $\pm$ standard deviation of the number of seeds for of <i>Z.mays</i> and <i>S. lycopersicum</i>	37
Figure 12 – Average values $\pm$ standard deviation of the fresh weight of <i>Z.mays</i> and <i>S. lycopersicum</i> plants.....	38
Figure 13 – Average values $\pm$ standard deviation of the dry weight of <i>Z.mays</i> and <i>S. lycopersicum</i> plants.....	38
Figure 14 – Average values $\pm$ standard deviation of the number of adults and reproductive activity of <i>F. candida</i> by the end of 28-day exposure.....	39
Figure 15 – Average values $\pm$ standard deviation of the reproductive activity of <i>E. fetida</i> by the end of 56-day exposure.....	40

List of abbreviations

OOMW – olive oil mill wastewater

EC – electrical conductivity

COD – chemical oxygen demand

BOD – biological oxygen demand

TSS – total suspended solids

Pt – total phosphorus

Nt – total nitrogen

OM – organic matter

CW – constructed wetland

SSFS – subsurface flow system

VF – vertical flow

HF – horizontal flow

VFCW – vertical flow constructed wetland

SWR – soil water repellency

CTL – control

E.75% – diluted OOMW

E.100% – undiluted OOMW

EC_x – effect concentration

S.CTL – substrate from CTL treatment

S.75% – substrate from E.75%

S.100% – substrate from E.100% treatment

RT – retention time

WHC – water holding capacity

LOEC – lowest observed effect concentration

NOEC – no observed effect concentration

LC_x – lethal concentration

1. Introduction

1.1. Olive oil production

Olive oil production, which worldwide exceeds 3 million tons per year, is a growing industrial activity (Table I) with a high economic importance mainly in the Mediterranean area, where more than 90% of the production is obtained (Aggoun et al., 2016; Azbar et al., 2010; Khdair & Abu-Rumman, 2020; Solomakou & Goula, 2021). Among the largest producers are Spain, Italy, Greece, and Turkey, followed by Morocco, Algeria, Tunisia, and Portugal (Ghadraoui et al., 2021; Khdair & Abu-Rumman, 2020; Yalcuk et al., 2010). In Portugal, the olive oil production is one of the most important economic activities that is carried out mainly by small family businesses (Martins et al., 2011). In recent years, the investment in intensive olive oil production has increased, mainly in the Alentejo region by around 64%, representing already 76% of national production. In this way, besides Portugal being considered self-sufficient in olive oil since 2014, its high production allows the opportunity to increase exports (Martins et al., 2011; República Portuguesa, 2021).

Table I – Olive oil production per campaign from 2016 to 2021. Source: International Olive Council, (2022).

Campaign	Olive oil production (tons)		
	Worldwide	European Union (EU)	Portugal
2016/17	2562000	1752000	69000
2017/18	3379000	2188000	135000
2018/19	3304000	2264000	100000
2019/20	3266000	1920000	141000
2020/21	3010000	2051200	100000

The extraction of olive oil is done following three steps: (i) olive crushing, where the fruit cells are broken down and the oil released; (ii) mixing, where the remaining paste is slowly mixed to increase the oil yield; and (iii) oil separation from the remaining wastes (Aggoun et al., 2016; el Ghadraoui et al., 2020; Solomakou & Goula, 2021). The separation of the residues can be carried out by one of the following methods: (i) traditional batch pressure process; or (ii) centrifuge extraction systems (Azbar et al., 2010; el Ghadraoui et al., 2020; Sierra et al., 2001). During these processes, water is added to wash the fruit and to allow greater production efficiency by extracting the maximum amount of oil (Bombino et al., 2021; Carrara et al., 2021; Herouvim et al., 2011). In the traditional process, after the extraction of oil from olives, the resulting pomace passes through pressure methods from which liquid residues composed of a mixture of water and oil are obtained, and after separated through vertical decantation (Azbar et al., 2010). In the case of the centrifuge

extraction systems they can be divided into two-phase processes, from which the oil and a solid-liquid mixture results, or three-phase processes, in which oil, solid residue and effluent are obtained (Bombino et al., 2021; Kataki et al., 2021; Khdair & Abu-Rumman, 2020). Compared to the traditional extraction method these processes have advantages, such as higher and better-quality of olive oil production, lower production costs, and the need for less space. However, using this type of processing there is a higher production of waste. For example, with the traditional process there is a production of 0.4-0.6 m³ of effluent and 500 kg of pomace per ton of olives, while the production of waste in centrifuge systems is estimated to be about 1.3 m³ of effluent and 580 kg of pomace for the three-phase system and 0.2-0.3 m³ of effluent and 730 kg of pomace in the two-phase system. Despite this, the two-phase system is still considered more environmentally friendly, since it requires less energy and less water and produces an effluent with less environmental burden, which comes mainly from washing the fruit, although it has the disadvantage of not separating the solid waste from the liquid, giving rise to a water-rich solid waste (Azbar et al., 2010; Khdair & Abu-Rumman, 2020; Solomakou & Goula, 2021). The two-phase systems are the most used in several Mediterranean countries, such as Spain, Italy, Portugal, and Greece (Azbar et al., 2010; Solomakou & Goula, 2021).

In this way, at the end of the production line, it will result the olive oil and two by-products a solid residue, composed of olive pulp and pits and a liquid residue, named olive oil mill wastewater (OOMW), which consists of a mixture of water, olive oil and olive solid residues (Campani et al., 2017; el Ghadraoui et al., 2020; Khdair & Abu-Rumman, 2020). The production of OOMW is estimated to be 0.5-1.5 m³ per 1000 kg of olives, depending on the extraction process used (Khdair & Abu-Rumman, 2020; Paraskeva & Diamadopoulos, 2006). And, although the olive oil production is done through processes that do not require the use of chemicals, the environmental concerns associated with the production of highly polluting wastes and effluents are increasing as olive oil production tends to keep growing worldwide (Achak et al., 2019; Aggoun et al., 2016; Kapellakis et al., 2012). It is estimated that about 5.4 x 10⁶ m³ of OOMW are produced annually, so its production has become a problem of high relevance in olive oil producing countries (Askari et al., 2022).

In general, OOMW is characterized by its intense dark colour, strong odour, acidic pH, high electrical conductivity (EC), and have in its composition high contents of organic matter (OM), complex and recalcitrant substances, long-chain fatty acids and phenolic compounds, with anti-microbial and phytotoxic properties (Table II) (Achak et al., 2019; Andiloro et al., 2021; Carrara et al., 2021). However, the characteristics of the waste resulting from olive oil production depend on several factors such as the type and maturity of the olives, the extraction method, soil properties, and weather conditions (Azbar et al., 2010; el Ghadraoui et al., 2020; Yalcuk et al., 2010).

Table II – Main composition of the olive oil mill wastewater (OOMW). Sources: 1Abd EL-Aziz et al., 2015; 2Achak et al., 2019; 3Asfi et al., 2012; 4Azbar et al., 2010; 5Bombino et al., 2021; 6Cabrera et al., 1996; 7Chartzoulakis et al., 2010; 8di Caprio et al., 2015; 9Ghadraoui et al., 2020; 10Ghadraoui et al., 2021; 11Khdair & Abu-Rumman, 2020; 12Masi et al., 2015; 13Paraskeva & Diamadopoulos, 2006; 14Sierra et al., 2001; 15Solomakou & Goula, 2021; 16Yalcuk et al., 2010.

	Range of values	Reference
pH	4.0 – 6.0	2,3,4,5,7,8,9,10,11,12,14,15
EC (mS/cm)	3.00 - 28.23	5,6,7,9,10,11,14
COD (g/L)	30 – 230	1,2,3,4,5,6,7,8,9,11,12,13,14,15,16
BOD (g/L)	15 – 135	2,4,6,11,13,14,15
TSS (g/L)	1.0 – 102.5	1,2,4,5,8,13
Pt (g/L)	0.020 – 1.1	10,11
Nt (g/L)	0.30 – 0.90	3,4,5,12
Phenolic compounds (g/L)	0.25 – 24.00	2,4,5,7,8,9,10,13,14

EC - Electrical Conductivity; COD - Chemical Oxygen Demand; BOD - Biological Oxygen Demand; TSS - Total Suspended Solids; Pt - Total Phosphorus; Nt - Total Nitrogen

The management of this waste becomes difficult due to the seasonality of production (2-4 months), the dispersion of the small industries, and the associated operational costs (el Ghadraoui et al., 2020; Kapellakis et al., 2012; Khdair & Abu-Rumman, 2020). These problems combined with the high organic content, complex and recalcitrant substances, as phenolic compounds present in its composition lead to major negative impacts associated with uncontrolled deposition and discharge of OOMW to land and water bodies. The repercussions are associated with adverse changes in soil structure and quality and subsurface infiltration, as well as problems related to seed germination inhibition and the antibacterial activity of OOMW (di Caprio et al., 2015; Ghadraoui et al., 2021; Masi et al., 2015). But also water pollution, causing problems such as oxygen depletion, eutrophication, development of surface grease films, and increased toxicity (Aggoun et al., 2016; Andiloro et al., 2021; Ghadraoui et al., 2021). Other environmental problems include contamination of groundwater, release of odours, and proliferation of mosquitoes (Bombino et al., 2021; Khdair & Abu-Rumman, 2020; Solomakou & Goula, 2021). However, despite the well-known negative effects associated with OOMW, the most common practice is the disposal and storage in evaporation ponds or in some cases the direct discharge into natural water bodies. Due to its high organic matter and nutrients content the OOMW has also been use for crop irrigation (Khdair & Abu-Rumman, 2020; Nogueira et al., 2015;

Solomakou & Goula, 2021). In some countries, the legislation already makes this practice available for agricultural purposes, for example in Portugal where it is possible to apply a maximum of 80 m³/ha/year (Dispatch no. 206/2000 of 6th June). However, several studies have been carried out in order to evaluate the application of different amounts of OOMW to the soil and its impact. In a study conducted by Sierra et al. (2007) with OOMW sourced from a stabilization lagoon, it was observed that the application of OOMW up to 360 m³/ha contributes to the increase of soil fertility, however, there was a temporary immobilization of nitrate and an increase in salinity and phenolic compounds, leading to the conclusion that a maximum application of 180 m³/ha/year would be recommended. Chartzoulakis et al. (2010) also studied the impact of raw OOMW irrigation on soil and observed that its application in controlled dosages contributes to soil fertility and that, due to the high amounts of organic matter (OM) and macronutrients, this effluent can be considered a good low-cost fertilizer. Campani et al. (2017) evaluate toxicological effects of the application of OOMW to the soil (doses: 12.5, 25 e 50% w/w) on the earthworm *Eisenia fetida* using several biochemical biomarkers. The results indicated that the use of OOMW can lead to the induction of oxidative stress and genotoxic effects even at lower doses probably due to the presence of high levels of phenols and that the application of high dosages caused earthworm's mortality. However, after a bioremediation treatment (composting/bioaugmentation process) a decrease in the toxicity of the OOMW was recorded. Asfi et al. (2012) also showed the negative impact of several dilutions of OOMW (1:10 and 1:20) on spinach plants development due to the low pH, high mineral salt content and high organic molecule content. Nonetheless, OOMW is a valuable resource and can be used as an agricultural fertilizer improving soil fertility, due to the presence of nutrients such as nitrogen, calcium, potassium and phosphorus in its composition. However, due to the presence of phenolic and lipid compounds, moderate pre-treatment and dosing is required in order to reduce the associated risks (Ayoub et al., 2016; Ghadraoui et al., 2021; Khdair & Abu-Rumman, 2020).

The methods for OOMW treatment fall into three groups: biological processes, which include aerobic and anaerobic treatments; physical and physicochemical processes, such as evaporation, reverse osmosis, thermal methods, oxidation, flocculation, and adsorption; or the combination of biological and physicochemical processes (Andiloro et al., 2021; Azbar et al., 2010; Khdair & Abu-Rumman, 2020). After passing through these treatments, OOMW valorisation as an economic resource can be considered in fields such as the biofuels industry (to produce biohydrogen, biodiesel and bio methanol), in agriculture (for composting or fungal biomass development), and for biochemical use (to obtain antioxidants, bioactive compounds, such as phenolic compounds, and other metabolites) (Andiloro et al., 2021; Khdair & Abu-Rumman, 2020; Solomakou & Goula, 2021). However, most of these methods are expensive, requiring high capital and operating costs, and insufficient, due to their limited efficiency in reducing the toxicity and organic content of the OOMW. In fact, this industry faces several challenges to deal with this waste (Azbar et al., 2010; Khdair & Abu-Rumman, 2020; Solomakou & Goula, 2021). Thus,

alternatives are needed for this industry, with the development of low-cost and easy-to-maintain treatments plants and methodologies, as for example those including nature-based solutions.

1.2. Constructed Wetlands

Constructed wetlands (CW) are low-cost effluent treatment alternatives, easily adaptable to more unfavourable socio-economic conditions, generally used for the treatment of urban effluents, but in recent years they also have been proposed and used to treat effluents of agro-food industries characterized by high levels of organic matter and nutrients (Calheiros et al., 2019; Kataki et al., 2021; Khdair & Abu-Rumman, 2020). These systems use natural processes involving substrate, plants, and the plants and substrate microbial community to mineralize and assimilate the pollutants and treat the effluent through physical (sedimentation and filtration), chemical (precipitation, ion exchange, volatilization, adsorption, and photolysis), and biological (aerobic and anaerobic mineralization, as fermentation and microbial immobilization and plant's uptake) processes (Calheiros et al., 2019; el Ghadraoui et al., 2020; Parde et al., 2021).

CW are characterized according to three main parameters: (i) hydrological dynamics, where they can be divided into open water-surface flow and sub-surface flow systems (SSFS); (ii) macrophyte type; and (iii) flow path, which can be vertical (VF) or horizontal (HF) in sub-surface wetlands (Parde et al., 2021; Vymazal, 2014; Yalcuk et al., 2010). In SSFS, the effluent flows through the substrate and macrophyte roots, undergoing physicochemical processes and microbiological degradation at anaerobic or aerobic conditions in the pores of the substrate materials (Kataki et al., 2021; Parde et al., 2021; Yalcuk et al., 2010). Thus, the filling media of CW is one of the most important components in the composition of these systems, contributing to the efficiency of physical, chemical and biological processes during the treatment. Thus, the substrate components should be selected following criteria that favour these processes. This is the case of gravel and sand, which can ensure the presence of pores for aeration of the substrate and surface area for the development of biofilms (el Ghadraoui et al., 2020; Kataki et al., 2021; Parde et al., 2021). In turn, macrophytes also play an important role in the treatment of effluents in the CW, since they assimilate pollutants directly into their tissues and promote the development of chemical and biochemical reactions in the rhizosphere zone, where they provide the right conditions for the development of the microbial community (Achak et al., 2019; Kataki et al., 2021; Parde et al., 2021). In the vertical flow CW (VFCW), a layer of gravel is usually followed by a layer of sand or soil in which the macrophytes are planted and through which the effluent percolates by gravity, creating an environment essentially aerobic, allowing a good maintenance of oxygen transfer and enables nitrification (Vymazal, 2014; Yalcuk et al., 2010).

Some advantages of these systems include the fact that they do not require sludge

treatment, they can remove pollutants and pathogens, have low maintenance costs and simple operational efforts and can tolerate variations in effluent production and its seasonality as well as a wide temperature range (Calheiros et al., 2019; Kataki et al., 2021; Khdair & Abu-Rumman, 2020). Also, generally, these systems exhibit high removal rates for organic matter (over 90% removal of BOD, COD and TSS), nitrogen (up to 98% removal) and phosphorus (up to 99% removal), depending on the type of system, flow regime, hydraulic conductivity, oxygen supply and chemical conditions of the substrate (Ayaz et al., 2012; Kataki et al., 2021; Khdair & Abu-Rumman, 2020). The reduction of organic matter is carried out by sedimentation, filtration, and biological degradation in the oxygenated layers. Regarding the nitrogen removal, it is done by assimilation by the macrophytes and microbiota present in the substrate and in the roots of the plants, and denitrification, depending on the oxygen supply. Phosphorus removal occurs by sedimentation and complexation, uptake by plants, and adsorption to the filling material, the latter depending on the surface area of the filling material and the pH of the medium, since in alkaline mediums and with materials rich in Ca, Al, Fe, or oxide groups the capacity for adsorption and precipitation of phosphorus is maximum (Achak et al., 2019; Ayaz et al., 2012; Parde et al., 2021). Achak et al. (2019) study the potential of a combined treatment with a sand filter and VFCW on the removal of organic matter and several nutrients from a diluted OOMW (1:1 diluted with domestic wastewater). The authors recorded an increase in pH and EC and high removal rates for COD (total and dissolved), ammonia, nitrates, total phosphorus and orthophosphates after the effluent passed through the sand filter. However, the VFCW whose substrate was composed of gravel and soil, showed a low removal capacity of organic matter and nutrients. And despite the efficiency of the combination of treatments (sand filter and VFCW) the OOMW treated did not reach the recommend limits established by the legislation for the discharge of this wastewater. While Ghadraoui et al. (2021) studied the effect of a mixture of OOMW with urban effluent on the physicochemical characteristics of the substrate (composed by sand, pozzolan and grave) of a VFCW, as well as the abundance and behaviour of the microbial community in the substrate. The results showed higher concentrations of EC, Nt, C/N ratio and polyphenols in the CW after the passage of the mixture and a significant increase of microbial community. Although several studies have been developed with high removal rates of pollutants, the efficiency of CW systems used was not enough to reach the limits recommended by the legislation to discharge this effluent, even when using dilution or pre-treatment of OOMW, so the use of CW could not yet be considered as a final solution for the treatment of this type of effluent.

1.3. Dissertation objective and structure

The main objective of this dissertation was to evaluate the efficiency of a OOMW treatment performed in laboratory scale VFCW unit, in improving its physicochemical properties, as well as

in reducing its toxicity to aquatic organisms. Furthermore, taking into consideration the seasonality of OOMW production and in a circular economy perspective, this study also aimed in assessing the safety of the CW substrate to be used as a natural soil amendment.

2. Material and methods

2.1. Pilot constructed wetland

Nine pilot CW units (three replicates per treatment) (Figure 1) were built on plastic boxes (56 cm width, 39 cm depth, 42 cm height) with a tap at the bottom to collect the OOMW after it has percolated by gravity through the substrate. A plastic net (5 mm pore) was placed at the bottom of each box, under the substrate to prevent clogging the effluent collecting tubes. The substrate was added in different layers: gravel as the first layer, to prevent clogging; expanded clay (LECA®) as second layer, to allow the development of biofilms and adsorb organic molecules, since this material has high surface are/volume ratio; and sand as third layer, for plant support.



Figure 1 – Schematic representation of the composition of the constructed wetland units and lab scale vertical flow constructed wetland.

The *Iris pseudacorus* plants were collected in Salreu (Ria de Aveiro, Portugal) and maintained at room temperature and natural photoperiod in buckets with water collected at the site before being planted in the CW units.

In each CW unit, 8 *I. pseudacorus* plants were planted and the system was allowed to stabilize for 4 weeks: in the first week the system was irrigated with natural water collected from Ria de Aveiro, and in the following 3 weeks the system was irrigated with a nutritive solution composed of 16 mL of nutrients per 1 L of deionized water (Hoagland solution: Table 1A of annexes). Before new additions, taps were opened to drain the excess of previously added water and to aerate the substrate. A stabilization period of 24 hours with each treatment was carried out.

2.1.1. Experimental Design

Three different treatments were prepared (each one with three replicates): a control (CTL), irrigated with Hoagland solution, to understand if CW substrate itself can change the quality of the physical-chemical parameters of the samples; the units receiving OOMW diluted with deionized water (E.75%) and the undiluted OOMW (E.100%).

In order to evaluate if the retention time (RT) needed to better treat the OOMW, three different RT were tested (48 h, 72 h and one week) for each treatment (CTL, E.75% and E.100%), adding 1 L of the corresponding effluent dilution or nutritive solution per unit, at each replicate.

By the end of each RT, samples of treated OOMW were collected, in a total of 9 samples (three replicates per treatment) and used for Microtox® assay (bioluminescence inhibition). Also, a composite sample of each treatment were obtained by combined the treated effluent collected at the 3 replicates of each treatment, which were used in the assays with *Raphidocelis subcapitata* (growth inhibition) and *Daphnia magna* (acute immobilization), always following the standard protocols OECD No. 201 (OECD, 2011) and OECD No. 202 (OECD, 2004), respectively. The same assays were also performed with a sample of the raw OOMW (not treated).

In all treatments, colour, pH, electrical conductivity (EC), total phosphorus (Pt), total nitrogen (Nt), and chemical oxygen demand (COD) were recorded in the collected sample, as well in the raw OOMW.

2.1.2. Physicochemical parameters

The pH of the samples was measured using a Hanna Edge® multiparameter with an HI11310 pH electrode, while EC was measured using an HI763100 electrode. Total phosphorus (Pt) and total nitrogen (Nt) were measured using the Reactor Hanna® C9800 and HI83399 multiparameter photometer, following adaptations of the EPA 365.2 method, using the ascorbic acid method for Pt, and the chromotropic acid method to determine Nt, according to the protocols delivered with the kits by the vendor. For the COD determination in the samples, the Reactors Hanna® HI839800 and HI83399 multiparameter photometer were used following an adaptation of the EPA 410.4 protocol for COD determination in wastewaters. The colour was measured by absorbance at wavelength associated with colored compounds (465 nm) using a UV/VIS spectrophotometer (Termo Fisher Scientific®).

2.1.3. Ecotoxicological assays

2.1.3.1. Bioluminescence inhibition assay with *Aliivibrio fischeri* (Microtox® assay)

The Microtox® assay was performed using a Microtox® Analyzer Model 500, which is a laboratory-based, temperature controlled and self-calibrating photometer that incubates the samples and the bacteria and registers the bacteria luminescence to measure acute toxicity. The test was performed using MicrotoxOmni software (AZUR Environmental, 1998), following the 81.9% Basic Test protocol, according to which the bacteria is exposed to various dilutions of the test sample (obtained with a dilution factor of 2) and the bioluminescence. The bacteria *A. fischeri* used in the assay was delivered lyophilized by the manufacture and rehydrated with a reconstitution solution right before starting the assay.

In this test, the intensity of bioluminescence of *A. fischeri* bacteria is measured before (I_0) and after 5, 15 and 30 minutes of exposure to several dilutions of the samples taken before and after each treatment (I_t). The variation of light intensity between I_0 and I_t and the concentration of the sample to which the bacteria are exposed produces a dose/response relationship, which can be translated into EC_x values (AZUR Environmental, 1998). The EC_x values and their corresponding 95% confidence intervals were computed using the MicrotoxOmni® software version V1.18 applying a linear model (AZUR Environmental, 1998).

2.1.3.2. *Raphidocelis subcapitata* growth inhibition test

In order to determine the toxicity of the raw and treated effluent on the growth of the freshwater green algae *R. subcapitata*, the assay was performed following the protocol OECD No. 201 (OECD, 2011), adapted for 24-well microplates (Geis et al., 2000). The microalgae was obtained from a laboratorial culture, kept under controlled conditions. Three days before the assays, around 3 mL of Woods Hole MBL with an microalgae inoculum obtained from the laboratorial culture were added to a sterilized Erlenmeyer with 75 mL of MBL medium (Table 3A of annexes) and incubated at continuous light and temperature ($21 \pm 1^\circ\text{C}$), to achieve the exponential growth phase before the assay. Before the assay, the number of cells in the prepared inoculum was counted using a Neubauer chamber to determine the concentration of the inoculum. The inoculum was after diluted with MBL medium to achieve a cell's density of 10^4 cells/mL. The samples collected from each treatment and retention time and the raw effluent sample were filtered with 1.5 μm pore nylon filter (Filter-lab® MFV6) to eliminate that have grown in CW units and tested 3 replicates of five dilutions between 100% and 19.8% (dilution factor of 1.5), under axenic conditions. A control sample only with MBL medium (used to dilute the effluent samples)

was also performed. The plates were placed in an orbital shaker under controlled conditions of temperature ($21\pm1^{\circ}\text{C}$) and continuous light for 72 hours. At the end of the test, the absorbance was measured at 440 nm and converted into cell/mL through a standard curve:

$$\text{Abs} = 5E^{-8}X + 0.0023$$

in which X is cell density. The average algae growth rate was calculated using the equation:

$$\text{GR} = \frac{\ln X_f - \ln X_i}{t_f - t_i} (\text{day}^{-1}),$$

in which GR is the average growth rate, X_i is cell density in the beginning of the assay, and X_f is cell density by the end of the assay.

2.1.3.3. Immobilization assay with *Daphnia magna*

To perform the *Daphnia* sp. acute immobilization test, the standard protocol OECD No. 202 (OECD, 2004) was followed. *Daphnia magna* neonates (with less than 24h) were obtained from laboratorial cultures of females kept under controlled conditions of $21\pm1^{\circ}\text{C}$ and a photoperiod of $16\text{h}^{\text{L}}:8\text{h}^{\text{D}}$, in 800 mL glass pots filled with ASTM hard water medium (Table 2A of annexes). Three times per week *D. magna* cultures were fed with *Raphidocelis subcapitata* and supplemented with an organic additive (*Ascophyllum nodosum*).

For the raw effluent several concentrations were tested ranging from 100% to 0.51%, using a dilution factor of 1.5 with ASTM medium. For the samples collected after each treatment only the 100% was tested. A control only with ASTM medium was also included. Three test replicates of each treatment were prepared in glass tubes with 25 mL and 5 neonates with less than 24 hours, from the 3rd to 5th broods were added. The tubes were kept under controlled conditions of temperature ($21\pm1^{\circ}\text{C}$) and photoperiod ($16\text{h}^{\text{L}}:8\text{h}^{\text{D}}$), for 48 hours. During the test period the organism were not fed. The number of immobilized organisms in each test tube was recorded after 24 and 48 hours of exposure.

2.2. Soil amendment with CW substrate – safety evaluation

2.2.1. Soil preparation and physicochemical properties

After all the treatments of the OOMW in the CWs, the gravel was discarded and the rest of the substrate components of the three replicates of each treatment (CTL, E75% and E100%) were mixed and crushed (to decrease the size of the LECA® balls) in order to obtain composite

samples: S.CTL, resulting from the three replicates that were irrigated with Hoagland's solution; S.75%, resulting from the three replicates that were irrigated with diluted OOMW; and S.100%, resulting from the three replicates irrigated with undiluted OOMW.

To assess the safety of the CW substrate as amendment, a natural soil collected from Campus Agrário de Vairão was used. The soil was defaunated (cycles of freezing at -20°C and drying at 40°C), dry and sieved at 4 mm to be used for ecotoxicological assays. The soil was amended with 5% of substrate of each treatment applied in the CWs and left to stabilize before physicochemical characterization. A total of four treatments were tested: Vairão (natural soil without amendment), S.CTL, S.75% and S.100%. Also, an artificial OECD soil composed by 70% of sand, 20% of kaolin clay, 10% of *Sphagnum* peat and pH of 6.5 ± 0.5 , was prepared and used as a control only to validate the assays (OECD, 2016a).

For the physicochemical characterization, the soil samples were sieved at 2 mm. The soil pH was measured in a soil-water suspension (1:5 w/v) following the method described by the standard protocol ISO 10390 (2005), in which the samples are allowed to shake for 1 hour, followed by 1 hour of rest before the measurements with the HI11310 pH probe. The same suspensions were left to rest for 24 h and the electrical conductivity (EC) was measured according to the protocol using a conductivity meter HI763100. The maximum water holding capacity (WHC_{max}) was determined according to the standard protocol ISO 11274 (2019), immersing plastic cups with the bottom replaced with filter paper and filled with the soil samples in a tray with water for 3 hours. After, the cups were removed from the water left draining in absorbent paper for 2 hours, to eliminate the excess of water, and weighted to record the saturated weight. The cups were placed in an oven at 105°C overnight, the dry weight was recorded, and the maximum WHC was calculated. The organic matter content (OM) of the samples was determined following the method described in the protocol SPAC (2000), by loss of ignition at 450°C for 8 hours.

Before the ecotoxicological assays, deionized water was added to the soil to adjust the WHC to 50% of its maximum value.

2.2.2. Ecotoxicological assays

2.2.2.1. Bioluminescence inhibition assay with *Aliivibrio fischeri* (Microtox® assay)

The toxicity of the soil amended with CW substrates was assessed evaluating the effect on the bioluminescence of the marine bacteria *A. fischeri*, following the Microtox® Basic Solid-Phase Test protocol and using the Microtox® Analyzer Model 500 (AZUR Environmental, 1998). For that, a suspension of each soil sample was prepared with the solid-phase diluent in a

liquid to solid ratio (m/v) of 1:5 and left stirring for 10 minutes. The bacteria was then exposed to several dilutions of each sample (obtained with a dilution factor of 2) and the bioluminescence measured after 5, 15 and 30 minutes. The EC_x values and their corresponding 95% confidence intervals were estimated as described in section 2.1.3.1., by adjusting the linear Probit model to the data (AZUR Environmental, 1998).

2.2.2.2. Seedling growth test with *Zea mays* and *Solanum lycopersicum*

The germination and the seedling growth test was performed according to standard protocol OECD No. 208 (OECD, 2006) to assess phytotoxicity of the soil amended with CW substrates on two crop species, *Zea mays* (monocotyledonous) and *Solanum lycopersicum* (dycotiledonous). The seeds were obtained from a local supplier and carefully inspected to discard those that were not in good condition.

The test pots (11.7 cm diameter, 6.5 cm height) used were previously holed in the bottom, and a cotton rope placed passing through the holes allowing the connection between the soil and a container with deionized water. Each pot was filled with 200 g of the respective soil sample (mixed or not with substrate, for more details please see section 2.2.1.) and twenty seeds of each test species were added and buried in the soil surface of each test pot. Five replicates of each treatment were prepared for each plant. At the beginning of the test, and only once, the treatments were irrigated with a commercial nutrient solution (Compo® NPK 7,5,6) taking in consideration the manufacture instructions. The pots were placed in a growth chamber under controlled temperature ($21 \pm 1^\circ\text{C}$) and a photoperiod of $16\text{h}^{\text{L}}:8\text{h}^{\text{D}}$. Pots were checked each day to count the germinated seeds and after 50% of the seeds germinated in the OECD soil replicates, the test was validated and started. Only the first 5 plants in each test pot were left to growth for 14 days. The pots were watered every two days with deionized water by adding water on the containers under the test pots.

By the end of the 14 days, the shoots of each replicate were cut and the fresh mass recorded. Afterwards, the fresh plants biomass per pot was dried at 60°C for 48 hours and the dry mass recorded.

2.2.2.3. Reproduction assay with *Folsomia candida*

The collembolans of the species *Folsomia candida* were obtained from laboratorial cultures kept under controlled conditions of $20 \pm 2^\circ\text{C}$ and a photoperiod of $16\text{h}^{\text{L}}:8\text{h}^{\text{D}}$. The collembolans were maintained in small plastic boxes with a medium composed of activated charcoal and plaster of

Paris (1:10), moistened with deionized water and fed with granulated yeast (OECD, 2016b).

The assay was carried out following the standard protocol OECD No. 232 (OECD, 2016b) under the same temperature and photoperiod conditions of culturing. Plastic pots were filled with 30 g of soil (mixed or not with the substrate from the above-described treatments, for more details please see section 2.2.1.) and 10 *F. candida* juveniles (9-12 days old juveniles) were added to each pot. Five replicates of each treatment were prepared. As a suitable food source, granulated yeast was added to each container twice a week during the assay, and the humidity of the soil adjusted. After 28 days of exposure, mortality and reproduction were assessed, counting the number of adults and juveniles in each replicate using the ImageJ software (Java, 2022) and the validity criteria checked for the replicates with OECD soil.

2.2.2.4. Reproduction assay with *Eisenia fetida*

The reproduction test with *E. fetida* was carried out to assess if the soil habitat function was compromised after the addition of the CW substrate, following the standard protocol OECD No. 222 (OECD, 2016a).

The earthworms were obtained from laboratorial cultures kept under controlled conditions at $20\pm 2^{\circ}\text{C}$ and a photoperiod of $16\text{h}^{\text{L}}:8\text{h}^{\text{D}}$. The organisms were maintained in plastic boxes filled with a medium composed of peat and defaunated horse manure (1:1), moistened with deionized water and weekly fed with rolled oat (OECD, 2016a). Before the assay, the earthworms were selected and left to acclimatize in Vairão soil and OECD soil for 48 hours.

The assay was performed in plastic containers (11.7 cm diameter, 13 cm height) under the same controlled light and temperature conditions as the cultures. All the replicates contained 500 g of soil and 10 worms with a fully developed clitellum weighing between 0.30 and 0.60 g. Five replicates of each treatment were prepared for the reproduction test. During the assay, organisms were fed once a week, with 5 g per box of defaunated horse manure, and the soil moisture content was weekly monitored and adjusted with deionized water. After 28 days of exposure, adult worms were removed from the test containers, leaving the cocoons in the soil for another 28 days. The adult worms removed were weighted in beginning and at the end of the exposure to record mass loss. At the end of the assay, the pots were placed in a water bath initially at a temperature of 40°C but rising to 60°C . By the end of a period of 20 minutes, the juveniles from each replicate appeared at the surface of the soil and were counted. The validity criteria was checked for the replicates of the CTL.

2.3. Statistical Analysis

Regarding the physicochemical parameters of the OOMW, a two-way analysis of variance (ANOVA) was performed to test for the significant effect of both factors, the effluent dilution and the retention time of the effluent in the CW, on pH, EC, Nt, Pt, COD and colour. When significant interactions ($p < 0.05$) between both factors were observed, differences among treatments were tested using one-way ANOVA followed by a Tukey multicomparision test. A one-way ANOVA followed by Tukey test was also employed to find out potential significant differences between physiochemical parameters of the CTL (nutritive solution) samples after each retention time. One-way ANOVAs followed by Dunnett 2-sided test was also applied to find out potential significant differences on the growth rate of the microalgae *R. subcapitata* between the control and tested dilutions of OOMW samples (E.75% and E.100%) before and after the CW treatment. No observed effect concentration (NOEC) and low observed effect concentration (LOEC) values were taken from ANOVA significant results ($p < 0.05$). For the growth inhibition test with *R. subcapitata*, the effect concentrations (EC_{10} and EC_{20}) and the 95% confidence limits were calculated using the non-linear least squares regression procedure supplied by the software package Statistica 8.0 (StatSoft, n.d.). The EC_{20} and EC_{50} values, with the 95% confidence limits, for *D. magna* immobilization exposed to the raw effluent were estimated by adjusting a Probit model to this binary.

Regarding the soil amended with the CW substrate, a one-way ANOVA followed by Dunnett 2-sided test was employed to find out potential significant differences in the endpoints assessed between the control (Vairão soil) and the treatments (S.CTL, S.75% and S.100%).

All the statistical analysis was made using IBM SPSS Statistics 26 software.

3. Results and discussion

3.1. Pilot constructed wetland

3.1.1. Physicochemical parameters

The physicochemical parameters of E.CTL were evaluated in order to determine if the substrate used in the CW units causes itself alterations in these parameters. It was possible to observe a significant increase of the pH ($F_{5,7}=16.54$; $p=0.006$) and EC ($F_{5,7}=15.99$; $p=0.007$) of the nutritive solution after passing through the CW (Figure 2) as function of the retention time, more pronounced in the case of the EC. However, no significant differences were observed in the remaining parameters ($p<0.05$). The increase of pH and EC after passing OOMW through two different natural calcareous soils with a retention time of 2 and 3 years was also reported in the study of Cabrera et al. (1996). This increase in alkalinity can be attributed to fixation of organic acids in the sand particles and release of carbonate salts from the CW substrate, as for the increase in salinity, this can be related to the release of ions from the substrate itself and mineralization of the organic matter (Achak et al., 2019; Ghadraoui et al., 2021).

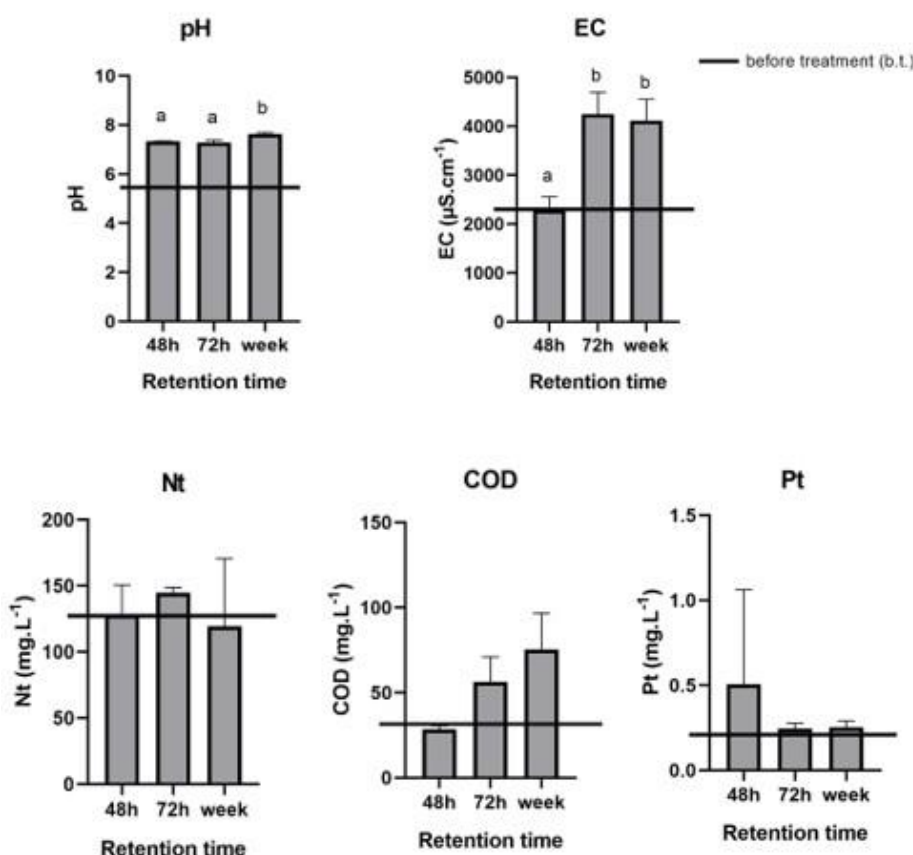


Figure 2 – Average values $\pm$ standard deviation of physicochemical parameters of E.CTL. The letters indicate significant statistical differences between retention times, $p<0.05$.

Furthermore, it was possible to observe high retention of the effluent on the substrate (up to 80%) as a function of the retention time (Figure 3). In this way, it can be possible to predict that if we increase the retention time, probably the effluent will be completely retained in the CW. This possibility of the total retention of the OOMW is favourable since if the treatment is not efficient enough to comply with the discharge limits of the treated effluent, it is not necessary to seek a final destination.

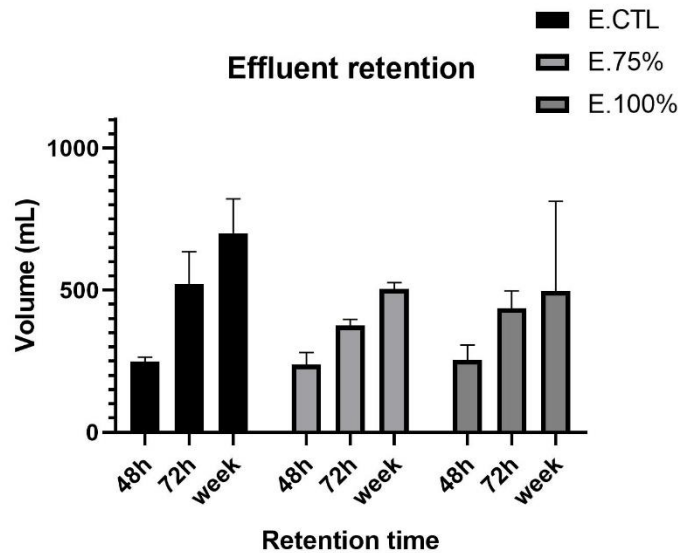


Figure 3 – Average values ± standard deviation of volume retention of E.CTL, E.75% and E.100%.

The evaluation of the physicochemical parameters of the raw and diluted OOMW (E.100% and E.75%, respectively) before and after treatment with different retention times was carried out in order to assess whether a longer retention time of the effluent in the CW units would improve the physicochemical properties of the samples. The results of the physicochemical parameters measurements of the OOMW before and after the treatments are presented in Figures 4 to 8. After passing the effluent through the CW, a significant increase in the pH was observed (Figure 4), registering significant differences for the RT and the %OOMW (RT: $F_{3,16}=159.33$, $p<0.0001$; %OOMW: $F_{1,16}=8.17$, $p<0.0001$), as well as an interaction between these two parameters ($F_{3,16}=4.45$, $p<0.0001$) which means that the variation of pH for each %OOMW depends on the RT. The effect was more pronounced after one-week of retention time for both diluted and undiluted OOMW (E.75%: $F_{8,11}=121.57$, $p<0.0001$; E.100%: $F_{8,11}=52.17$, $p<0.0001$). These results are in agreement with the results obtained by Achak et al. (2019). In this work a diluted sample of OOMW (1:1 with a domestic wastewater) was pre-treated by sand filters followed by VFCW. The substrate of the CW was composed by gravel and soil, and a mixture of plants (*Phragmites australis*, *Typha latifolia* and *Arundo donax*), and a residence time of 2 days. Cabrera et al. (1996) also observed an increase in alkalinity after passing OOMW through lysimeters filled

with natural calcareous soils for a period of 2 and 3 years. This increase may be caused by the release of carbonate salts from the substrate and by the fixation of organic acids in the substrate (Achak et al., 2019).

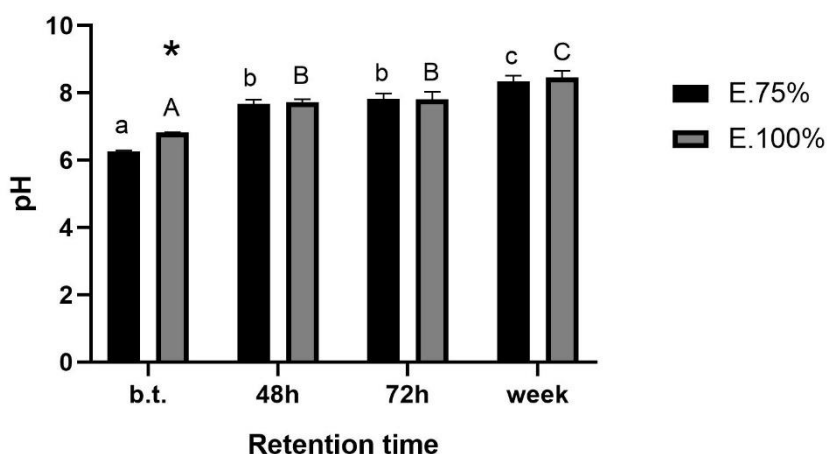


Figure 4 – Average values $\pm$ standard deviation of pH. Lower case letters indicate statistically significant differences between RT on diluted OOMW; upper case letters indicate statistically significant differences between RT on raw OOMW. * Indicates significant differences between diluted OOMW and raw OOMW ($p < 0.05$). b.t. – Sample before treatment.

As for the EC, a significant increase in salinity was recorded in the samples after passing through the CW substrate (RT: $F_{3,16}=188.93$, $p < 0.0001$; %OOMW: $F_{1,16}=44.75$, $p < 0.0001$) also with an interaction between RT and %OOMW ($F_{3,16}=18.30$, $p < 0.0001$), allowing to assume that the increase of EC depends on RT for both raw and diluted OOMW. A more pronounced increase was recorded for the diluted effluent (E.75%) at the end of the retention times of 72 h and one week ($F_{8,11}=335.97$, $p < 0.0001$). These results are also in accordance with the studies of Achak et al. (2019) and Cabrera et al. (1996). The increase in salinity may be related to the release of ions from the degradation of organic compounds, mineralization of OM, wear of the substrate itself, or saturation of the ion fixation sites (Achak et al., 2019; Ghadraoui et al., 2021).

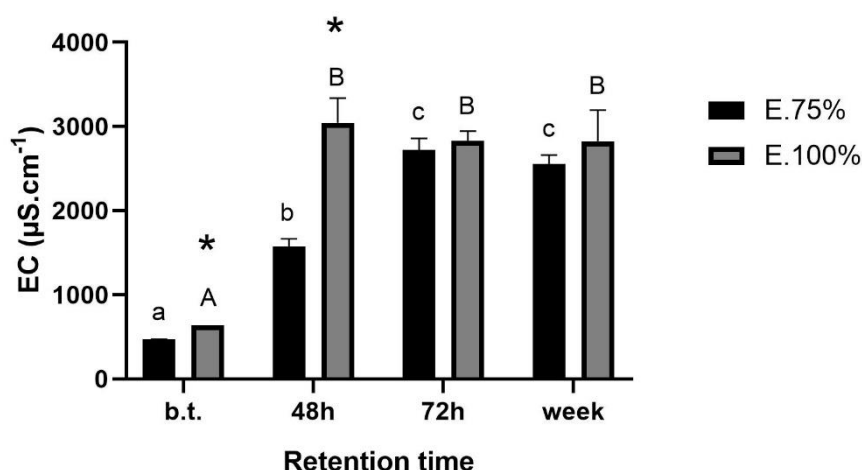


Figure 5 – Average values $\pm$ standard deviation of electrical conductivity (EC). Lower case letters indicate statistically significant differences between RT on diluted OOMW; upper case letters indicate statistically significant differences between RT on raw OOMW. * Indicates significant differences between diluted OOMW and raw OOMW ($p < 0.05$). b.t. – Sample before treatment.

Regarding Nt (Figure 6), a significant interaction between RT and % OOMW was recorded ($RT \times \%OOMW$: $F_{3,16}=3.64$, $p=0.036$), however only significant differences were observed between the RT ($F_{3,16}=23.96$, $p<0.0001$) since an increase were recorded after the 48 h of retention in the CW (E.75%: $F_{8,11}=30.41$, $p<0.0001$; E.100%: $F_{8,11}=4.08$, $p=0.050$), nevertheless the concentration of total nitrogen decrease when we increase the RT. In fact, for the diluted effluent after 72 h of RT the Nt reach the same values as the sample before treatment. However, in most of the studies, the results obtained report the reduction of Nt, with high removal rates, as recorded in the study of Cabrera et al. (1996) that observed a removal rate of up to 99.87% after passing OOMW through calcareous soils. Also Herouvim et al. (2011) recorded a removal rate for Nt of 75% after passing pre-treated OOMW through VFCW with a substrate composed of sand and gravel with *Phragmites australis* plants. In the work of Kapellakis et al. (2012) with diluted OOMW with fresh water (1:10) in free water surface CW with *Phragmites australis* plants and a substrate composed by gravel, the results showed also high removal rates for Nt (up to 87%). In our case, the recorded increase in Nt may be related to the presence of plants in the substrate and to the microbial activity during the substrate stabilisation period before OOMW passage.

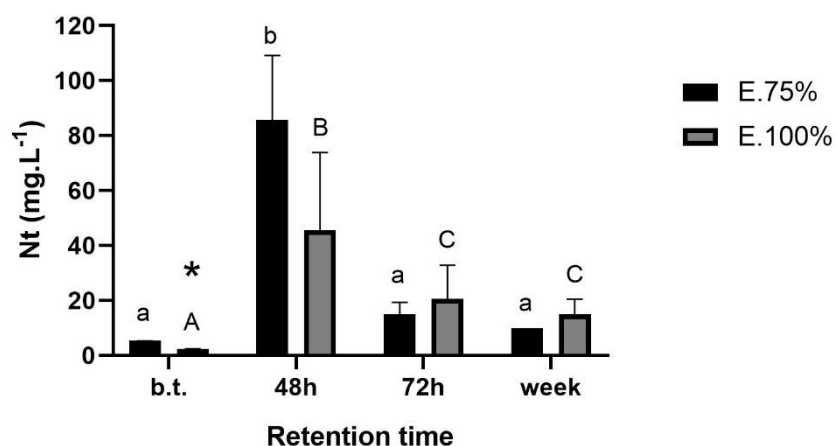


Figure 6 – Average values $\pm$ standard deviation of total nitrogen (Nt). Lower case letters indicate statistically significant differences between RT on diluted OOMW; upper case letters indicate statistically significant differences between RT on raw OOMW. * Indicates significant differences between diluted OOMW and raw OOMW ($p < 0.05$). b.t. – Sample before treatment.

Regarding Pt and COD, a significant decrease was recorded (Pt: $F_{3,16}=60.97$, $p < 0.0001$; COD: $F_{3,16}=542.83$, $p < 0.0001$) after the treatment in the CW units regardless the retention time (48 h, 72 h or 1 week) or the %OOMW (Figure 7) with Pt and COD removal rates of up to 78.71% and 98.10%, respectively. Other authors have also reported these decreases in their work, as reported in the work of Cabrera et al. (1996), with removal rates of up to 99.92% and 99.98 for COD and Pt, respectively, as well as reported by Kapellakis et al. (2012), with up to 90% for COD and 85% for Pt. Herouvim et al. (2011) also reported removal rates of 70% for COD and 87% for orthophosphates. The reduction of Pt may be related to phenomena of uptake by plants, adsorption of P on the substrate and sedimentation or complexation. As for COD, its reduction can be related to the mineralization of organic compounds during the treatment (Achak et al., 2019; Ayaz et al., 2012; Kapellakis et al., 2012).

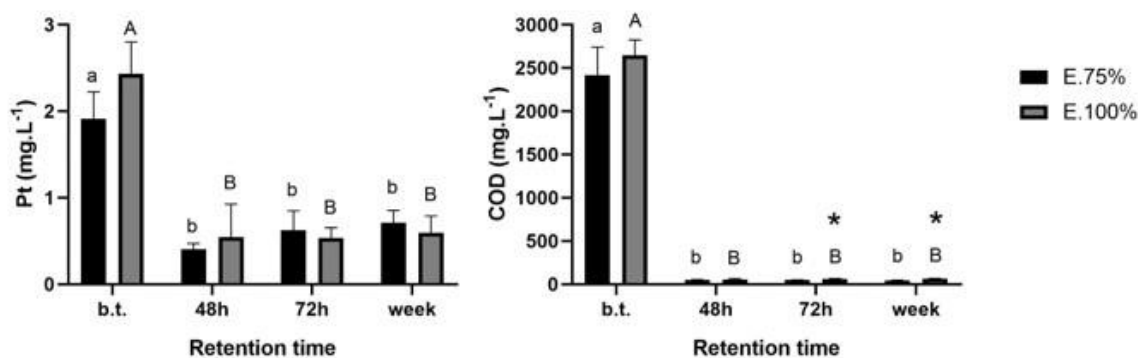


Figure 7 – Average values $\pm$ standard deviation of chemical oxygen demand (COD) and total phosphorus (Pt). Lower case letters indicate statistically significant differences between RT on diluted OOMW; upper case letters indicate statistically significant differences between RT on raw OOMW. * Indicates significant differences between diluted OOMW and raw OOMW ($p < 0.05$). b.t. – Sample before treatment.

For the colour of the effluent before and after the treatment, it was possible to observe that after the treatments in the CW units a significant decrease in the absorbance was recorded (RT: $F_{3,16}=1744.70$, $p<0.0001$; %OOMW: $F_{1,16}=43.63$, $p<0.0001$) as function of RT, since a significant interaction between RT and %OOMW was recorded ($F_{3,16}=39.63$, $p<0.0001$), with up to 99.17% of removal efficiency. However, a slight increase (not statistically different) in the colour of the samples was observed at the one-week retention time compared to samples taken at the 48 h and 72 h retention times. The increase in colour at the highest retention time may be related to the fact that the environment in the substrate became favourable for algal growth as the CWs were not protected from light and the nutrient input through the effluent was high.

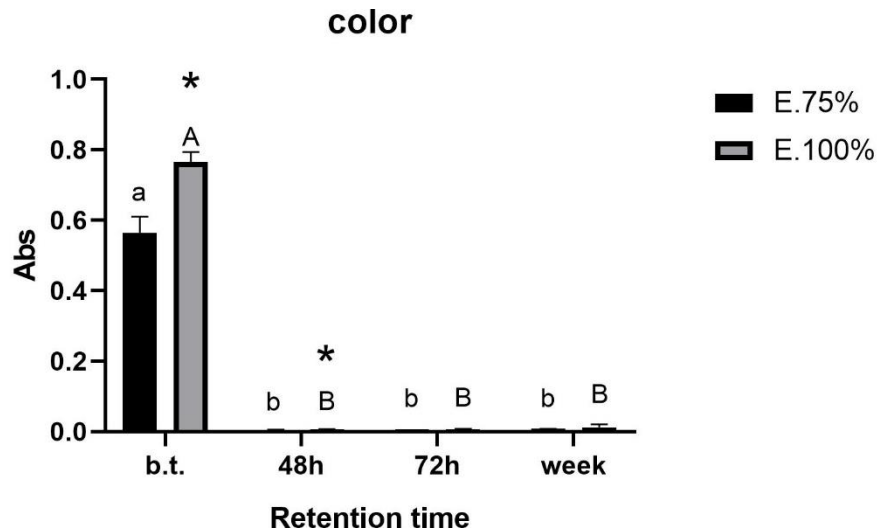


Figure 8 – Average values $\pm$ standard deviation of the colour. Lower case letters indicate statistically significant differences between RT on diluted OOMW; upper case letters indicate statistically significant differences between RT on raw OOMW. * Indicates significant differences between diluted OOMW and raw OOMW ($p<0.05$). b.t. – Sample before treatment.

In Table 4A of annexes is presented a summary of the observed effects on the physicochemical parameters of both raw and diluted OOMW as a function of the retention times.

3.1.2. Ecotoxicological assays

3.1.2.1. Bioluminescence inhibition assay with *Allivibrio fischeri* (Microtox® assay)

The results obtained in the Microtox® test, showed that the nutritive solution is not toxic for *A. fischeri*, however, a high toxicity of the raw OOMW was observed, recording an EC_{20} of 1.61% and an EC_{50} of 10.4% after 30 minutes of exposure. Also, a high toxicity of the diluted OOMW

was recorded with an $EC_{20-30 \text{ min}}$ of 1.24% and an $EC_{50-30 \text{ min}}$ of 15.27%. These results are in accordance with the results obtained by Nogueira et al. (2015) with a 50% diluted OOMW, that also found *A. fischeri* to be highly sensitive, recording an $EC_{50-30 \text{ min}}$ of 1.82%. Babić et al. (2019) also showed the high toxicity of a raw OOMW sample recording an $EC_{50-30 \text{ min}}$ of 0.24%. According to the authors this toxicity may be related to metabolic disturbances caused by the acid pH of the raw effluent and the presence of phenolic compounds and long chain fatty acids in its composition (Babić et al., 2019). However, after the passage of the OOMW effluent on the CW no toxicity was recorded, even for the non-diluted OOMW (E.100%) and a low residence time (only 48 hours). These results may be related with the degradation of the phenolic compounds and long-chain fatty acids of the raw OOMW and the increase of alkalinity of the effluent after passing through the CW substrate. This allowed to corroborate the high efficiency of the treatments in the CW units for the reduction of the toxicity of OOMW to *A. fischeri*.

3.1.2.2. *Raphidocelis subcapitata* growth inhibition test

The effects of the untreated OOMW on *R. subcapitata* growth after 72 h of exposure are represented in Figure 9. *R. subcapitata* showed a high growth inhibition when exposed to untreated OOMW ($F_{12, 19}=329.72$, $p<0.0001$) recording an EC_{20} of 27.94% (9.85%-46.04%) and an EC_{50} of 62.51% (41.26%-83.76%). Babić et al. (2019) also found that the green microalgae *Chlorella vulgaris* is highly sensitive to raw OOMW, recording an EC_{50} of 5.20% (3.56%-7.60%). This toxic effect on the microalgae may be related with the phenolic compounds contained in the raw OOMW, as showed in the study of Babić et al. (2019), in which the polar fraction of OOMW, mainly composed by phenolic compounds, was tested.

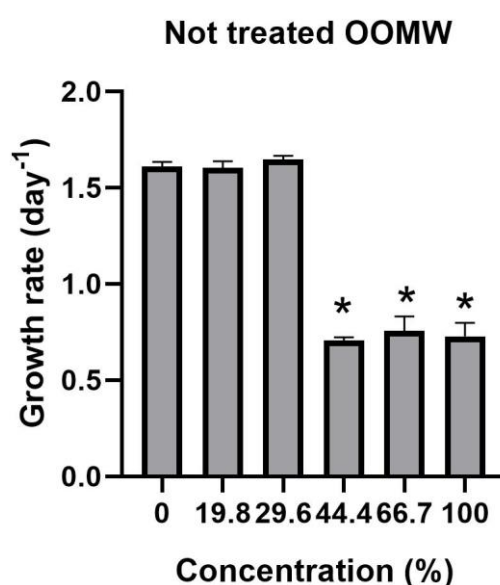


Figure 9 – Average values $\pm$ standard deviation of *R. subcapitata* growth rate exposed to several dilutions of raw OOMW (E.100%).

* Indicates significant statistical differences from the control (MBL), $p<0.05$.

After passage of the effluent in the CWs, it was possible to observe a reduction in toxicity in the samples when compared to the toxicity recorded for the untreated effluent, as can be seen in Figure 10. For the E.100% sample no toxicity was recorded independent of the RT in the CW, however a significant stimulation in the growth rate of *R. subcapitata* was recorded for all (48 h: $F_{12, 17} = 18.36$, $p < 0.0001$; 72 h: $F_{12, 17} = 10.29$, $p = 0.001$; week: $F_{12, 17} = 6.25$, $p = 0.004$). As for the E.75%, a significant inhibition on the growth rate was recorded in the samples collected after 48 h and 72 h (48 h: $F_{12, 17} = 67.84$, $p < 0.0001$; 72 h: $F_{12, 17} = 316.76$, $p < 0.0001$). An EC_{10} of 42.09% (23.08%-61.10%) and an EC_{20} of 81.77% (62.42%-101.11%) were recorded for the 48 h samples, and an EC_{10} of 86.32% (72.07%-100.58%) and an EC_{20} of 93.27% (85.82%-100.73%) for the 72 h. This inhibition may be related with the release of allelopathic substances by the plants used in the CW (Calheiros et al., 2019). However, increasing the RT in the CW the toxicity of the samples reduces, as can be seen by the recorded EC values for the 48 h and 72 h samples, and after one week of RT no toxicity was observed. The reduction in toxicity as a function of retention time may be related to the ability of the phenolic compounds present in the OOMW composition to adsorb or transform upon their passage through the CW substrate (Babić et al., 2019). This reduction may also be related with the retention of the allelopathic substances in the substrate itself (Calheiros et al., 2019). Concerning the stimulation in the growth of the microalgae, this effect may be related with the high nutrient load, especially N, of the samples.

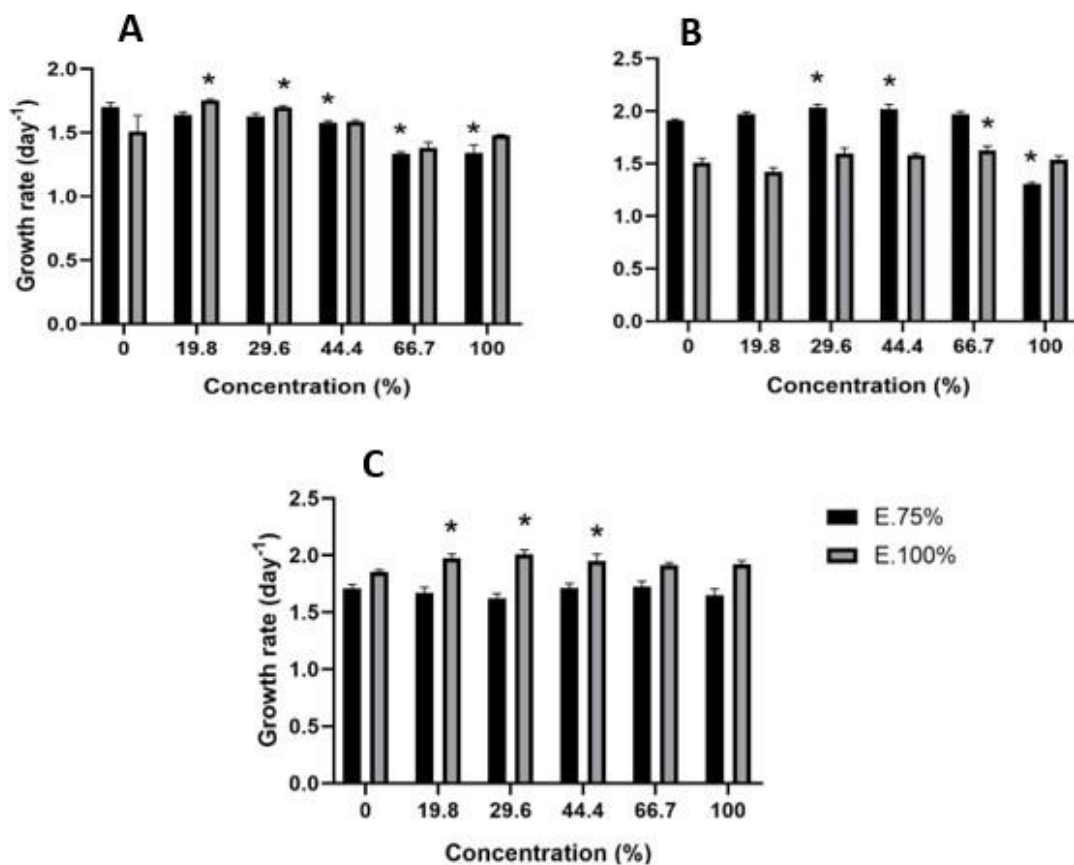


Figure 10 – Average values $\pm$ standard deviation of *R. subcapitata* growth rate exposed to several dilutions of samples after 48h (A), 72h (B) and one week (C). * Indicates significant statistical differences from the control (MBL), $p < 0.05$.

3.1.2.3. Immobilization assay with *Daphnia magna*

The acute test performed with the raw effluent showed high toxicity. An EC_{50} of 5.30% was detected in the 24 h exposure, and an EC_{50} of 2.67% (2.13-3.35) for the 48h exposure. These results are in accordance with the ones obtained in the study conducted by Babić et al. (2019) where an EC_{50} of 1.43% (1.33%-1.55%) was obtained for *D. magna*. This toxic effect may be related with the phenolic load of OOMW, has studied by Babić et al. (2019), that observed similar toxic effects when exposed *D. magna* to the polar fraction of OOMW. However, the samples of OOMW collected after the treatments in the different retention times showed no acute effects on *D. magna*, demonstrating the CW ability to reduce the toxicity of this effluent probably due to the removal of the phenolic compounds during the treatment.

3.1.3. Overall assessment of the system's efficiency

As already mentioned, VFCWs have been used in the treatment of OOMW and their efficiency has been studied. For example, in the work of Cabrera et al. (1996), lysimeter experiments were carried out in two boxes (C1 and C2) with two different natural calcareous soils (S1 and S2) to study the removal capacity of organic and inorganic pollutants from OOMW over a period of 2 and 3 years. A third box (C3) was prepared with S2 soil to be used to treat the leachates collected from C2. The results obtained showed that after the OOMW infiltration into the soils there was an increase in pH and EC of the effluent and removal rates of 99.92% of COD, 99.92% of BOD, 99.87% of Nt and 99.99% of Pt were observed. In another work performed by Herouvim et al. (2011), the efficiency of VFCW with a substrate composed of sand and gravel, and with *Phragmites australis* plants was used for the treatment of OOMW over 7 months. The OOMW used went through a pre-treatment in a biological filter, which retains part of the organic content and some nutrients. Removal rates of about 70% of COD, 75% of Nt and 87% of PO_4^{3-} were recorded. Another study, conducted by Kapellakis et al. (2012), assessed the treatment of diluted OOMW with fresh water (1:10) in a free water surface CW with *P. australis* plants and a substrate composed by gravel. The results showed a removal rate up to 90% of COD, 78% of Nt, and 80% of Pt. However, it was reported that the pollutant concentration in the effluent exceeded the limits imposed by legislation for discharge in water bodies. Achak et al. (2019) studied the removal of N and P from OOMW by combining sand filters followed by VFCW with a substrate composed by soil and gravel, and with a mixture of aquatic plants (*Phragmites australis*, *Typha latifolia* and *Arundo donax*) with a residence time of 2 days. The study was carried out with OOMW previously filtered and diluted with domestic wastewater (1:1). After passing the effluent through the system, removal rates of 62.48% for Nt, 90.43% for NH_4^+ , 77.25% for NO_3^- , 98.51% for PO_4^{3-} , 97.53% for Pt, and 99.05% for total COD were achieved. In the work conduct by Ghadraoui et al. (2021), with the aim to evaluate the effects of OOMW and urban wastewater on CW substrate

physicochemical parameters, the authors used a mixture of OOMW and urban wastewater in different proportions. The CWs were prepared with layers of sand, pozzolan and gravel, and with *Phragmites australis* plants during a period of 1 year. The authors reported removal rates of 91%, 94%, 94%, 92% and 95% for COD, PO_4^{3-} , Pt, NO_2^- , and NH_4^+ , respectively. In this work, the improvement of physicochemical properties of undiluted OOMW was observed after passing through the CW substrate composed of sand, expanded clay and gravel. A significant increase in alkalinity of the effluent as well as removal rates up to 78.71%, 98.10% and 99.17% for Pt, COD and colour, respectively, were recorded, without a need of dilution and using a low retention time (48h). After passage through the CW substrate, the pH and Pt values of the treated OOMW were within the values established by Portuguese legislation (Ministério do Ambiente, 1997) (pH: 6.0-9.0; Pt_{max} : 2mg/L). However, for Nt and COD the values did not reach the limits established by the legislation (Nt_{max} : 15mg/L; COD: 25mg/L O_2). In addition, a high percentage of effluent volume retention (up to 80%) was also observed, which allows predicting that the increase in RT can contribute to total retention of OOMW, making it unnecessary to seek a final destination for the effluent if it does not meet the legislated limits after treatment at CW. Furthermore, the decrease in toxicity after treatment of OOMW in CW could be recorded for the tested organisms.

3.2. Soil amendment with CW substrate – risk assessment

3.2.1. Physicochemical parameters

The physicochemical parameters of the soil samples are presented on Table III. The analysis of the samples showed that the soil correction with the CW substrate does not cause considerable changes in the physicochemical properties of the soil. However, several studies conducted to assess the effect of soil irrigation with OOMW already show that this application can change some soil properties. In the studies of Sierra et al. (2001, 2007), with the aim to evaluate the effects of irrigation of a calcareous nature soil with raw OOMW at agronomic rates (30, 180 and 360 m^3/ha) recorded considerable increase of soil pH proportional to the rate applied. This increase was related with the formation of carbonate compounds. The study of Hentati et al. (2016) to assess the effects of soil contamination with olive oil wastes, showed a considerable increase in EC related to ions (K^+ , Cl^- , SO_4^{2-} , PO_4^{3-} and NH_4^+) present on the wastes, that can cause perturbances in plants and organisms if the salinity is too high. Increases in the soil salinity may be related to the release of ionic species such as sulphate and potassium chloride, by OOMW into the substrate or by mineralization and transformation of nitrates present in OOMW that are retained in the substrate (Hentati et al., 2016; Sierra et al., 2001). The decrease in WHC recorded in this work may be related to the presence of lipid compounds retained in the substrate after the passage of the OOMW (Babić et al., 2019).

Table III – Physicochemical characterization of soil samples.

Treatment	Parameters				
	pH _{water}	pH _{KCl}	EC (μS/cm)	WHC (%)	OM (%)
OECD	5.64±0.05	5.05±0.15	141.07±10.95	30.07±0.31	7.59±0.22
Vairão	6.42±0.07	6.61±0.15	103.60±10.19	35.46±0.33	6.93±0.30
S.CTL	6.52±0.02	6.25±0.08	97.77±3.76	32.44±1.29	5.97±0.28
S.75%	6.60±0.01	5.96±0.07	81.53±2.16	32.01±1.08	6.05±0.30
S.100%	6.57±0.03	5.78±0.04	107.43±1.46	31.81±1.67	5.92±0.08

3.2.2. Ecotoxicological assays

3.2.2.1. Bioluminescence inhibition assay with *Allivibrio fischeri* (Microtox® assay)

Table IV records the EC₅₀ values observed at 5, 15 and 30 minutes of exposure of *A. fischeri* bacteria to the soil samples. According to the classification used by Kwan & Dutka (1995), the samples did not present any toxicity, since the EC₅₀ value is greater than 10,000 mg/L in all samples, confirming that the correction of the soil with the CW substrates does not cause negative disturbances for the soil microbiota.

Table IV – EC₅₀ values recorded after 5, 15 and 30 minutes of exposure of the bacteria *A. fischeri* to the samples of soil amended with the different substrates.

Treatment	EC ₅₀ (mg/L)		
	5 min	15 min	30 min
Vairão	48900	92341	140602
S.CTL	210646	942289	1944734
S.75%	31332	52522	64148
S.100%	371438	6800566	1732805

Although in our study no toxicity was recorded for the bacteria *A. fischeri* several studies already reported the antibacterial properties of the OOMW on soil microbial community. Piotrowska et al. (2006) studied the use of OOMW for agricultural soil fertigation in a rate of 80 m³/ha in a laboratory model system. The OOMW applied in the soil was previously centrifuged and filtered. The amended soil was kept in the dark for 14, 28 and 42 days and soil microbial and enzymatic analysis were performed. The obtained results showed changes on the biochemical parameter after 14 days of incubation. However, most of the parameters returned to their initial values after 28 days. The authors conclude that after the removal of the phenolic compounds, the application of OOMW for soil fertigation in semiarid areas may be a good option. Mekki et al.

(2006) also assess the effect of raw and biologically treated OOMW irrigation on soil properties and microbial communities. The results showed increased respiration on the soil samples amended with OOMW and an increased total mesophilic number in the treated OOMW, while the number of nitrifiers and fungi decreased. Toxic effects were recorded for the untreated OOMW from 100 m³/ha irrigation, and the toxicity was more significant with the 200 m³/ha application.

3.2.2.2. Seedling growth test with *Zea mays* and *Solanum lycopersicum*

Throughout the seedling growth test, the number of germinated seeds for *Z. mays* and *S. lycopersicum* seeds was recorded (Figure 11) and the results showed that no significant differences were observed between the control (Vairão soil) and the soil amended with the different substrates for both plant species (*Z. mays*: $F_{16, 19}=1.94$, $p=0.164$; *S. lycopersicum*: $F_{16, 19}=0.96$, $p=0.434$). For the shoot fresh biomass (Figure 12), also no significant differences were observed for both plants (*Z. mays*: $F_{16, 19}=1.36$, $p=0.289$; *S. lycopersicum*: $F_{16, 19}=1.35$, $p=0.295$). However, concerning the dry biomass (Figure 13), a significant stimulation for *S. lycopersicum* plants ($F_{16, 19}=2.82$, $p=0.072$) between the control and S.100% ($p=0.038$) was observed, but no significant differences for *Z. mays* plants ($p>0.05$). This increase on *S. lycopersicum* plants dry biomass may be related with nutrients present in the OOMW that remained adsorbed in the substrate.

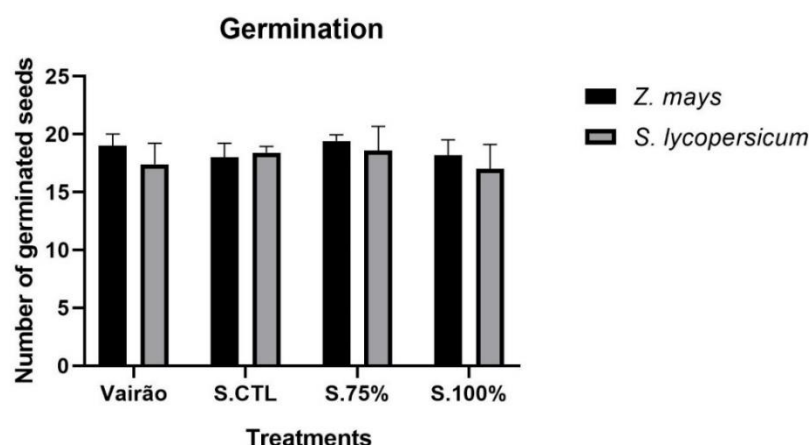


Figure 11 – Average values $\pm$ standard deviation of the number of seeds for *Z.mays* and *S. lycopersicum*.

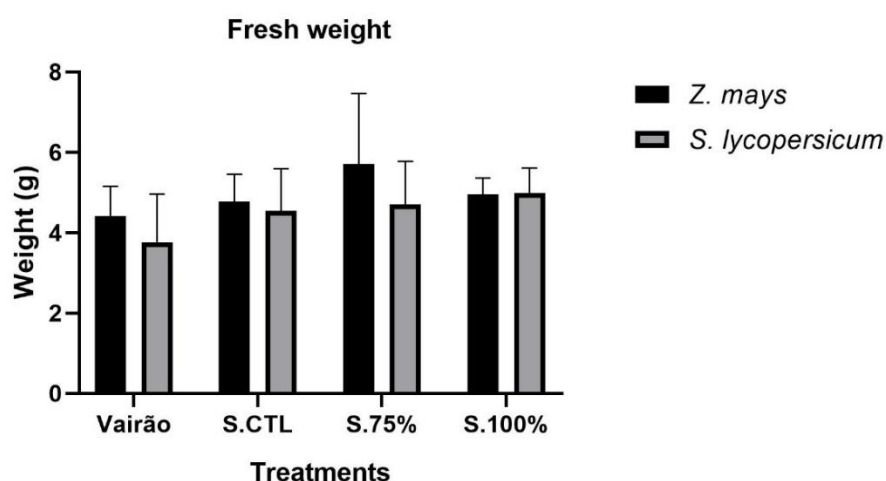


Figure 12 – Average values $\pm$ standard deviation of the fresh weight of *Z.mays* and *S. lycopersicum* plants.

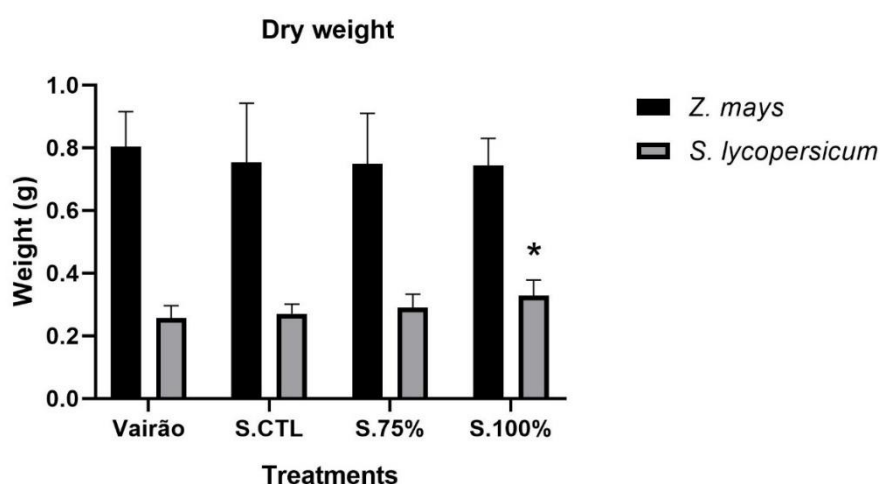


Figure 13 – Average values $\pm$ standard deviation of the dry weight of *Z. mays* and *S. lycopersicum* plants. * Indicates significant statistical differences from the control (Vairão soil), $p < 0.05$.

Babić et al. (2019) studied the impact of soil irrigation with raw OOMW in *Trifolium repens* and *Triticum aestivum* crops and the results showed toxic effects on seed germination (*T. repens*: $EC_{50}=8.68\%$; *T. aestivum*: $EC_{50}=11.58\%$), that may be related with the osmotic and ionic toxicity of the salts present on OOMW composition. Hentati et al. (2016) also studied the impact of soil amended with olive oil wastes in the cherry tomato *Lycopersicon esculentum* and observed a significant decrease of fresh biomass with increasing concentrations of olive oil wastes ($EC_{20}=0.293\%$, $EC_{50}=0.6\%$). In our work, no phytotoxicity was recorded, which allows us to affirm that the use of substrates in the soil is a better alternative to the direct application of OOMW or olive oil mill wastes. In the study of Hanafi et al. (2011) the value of OOMW as fertilizer was

assessed using an effluent treated by electrocoagulation followed by a biological treatment with *Aspergillus niger*. The phytotoxicity of the effluent was assessed before and after the treatments through a germination assay with *Triticum durum* seeds. The results showed that the raw effluent was very toxic for the seeds since the germination index was 0%. However, after the treatments, the phytotoxic effect reduced as the concentration of organic and phenolic compounds decreased.

3.2.2.3. Reproduction assay with *Folsomia candida*

The results of the reproduction assay with *F. candida* showed that the amendment of the soil with the CW substrates did not translate into disturbances in the survival of *F. candida* adults ($F_{16,19}=1.88$, $p=0.173$). Regarding the reproductive activity, only the soil amended with S.CTL showed significant differences from the control ($F_{16,19}=8.83$, $p=0.001$), with an increase in the number of juveniles (Figure 14).

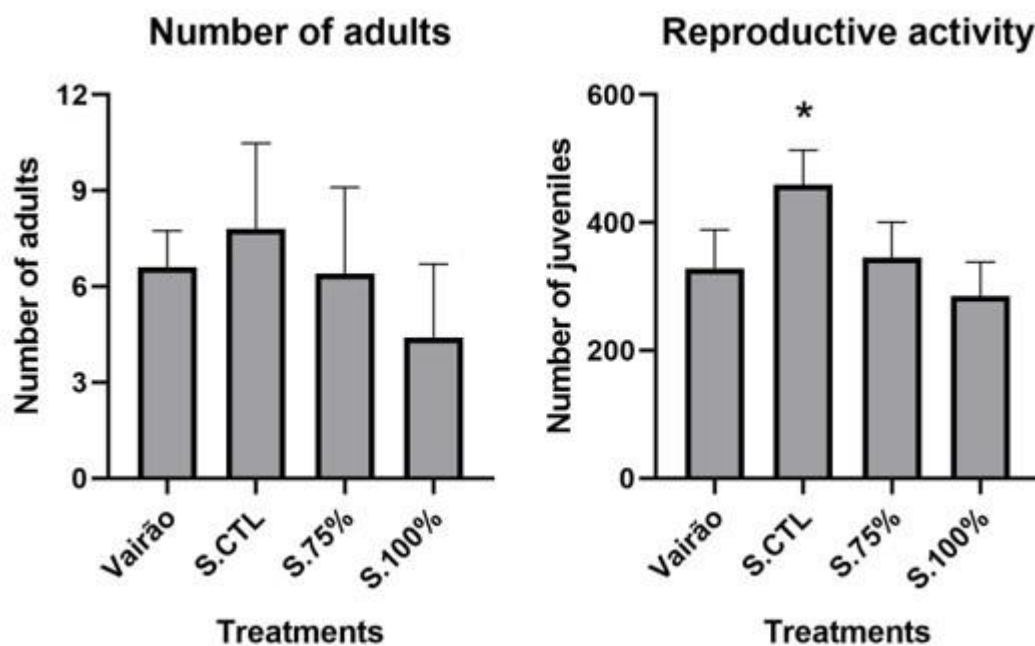


Figure 14 – Average values $\pm$ standard deviation of the number of adults and reproductive activity of *F. candida* by the end of 28-day exposure. * Indicates significant statistical differences from the control (Vairão soil), $p<0.05$.

Kovacevic et al. (2022) study the impact of OOMW and olive oil mill waste contaminated soil on *F. candida*. The OOMW and the contaminated soil was mixed with the artificial OECD soil at several ratios (0, 3.125, 6.25, 25 and 50%) and negative impacts on survival ($LC_{50}=32.34\%$ for OOMW and $LC_{50}=45.36\%$ for the contaminated soil) and reproduction ($EC_{50}=10.10\%$ for OOMW and $EC_{50}=19.44\%$ for the contaminated soil) of *F. candida* were observed after exposure. Other negative effects, such as neurotoxicity, oxidative stress and changes in available energy were also recorded. These negative effects may be related with the presence of phenolic compounds

in OOMW and the contaminated soil, showing that the direct irrigation of soil with this effluent presents high toxicity to soil organisms. Another study performed by Hentati et al. (2016) assess the effects of olive oil mill wastes on the habitat function performing an avoidance assay with *F. candida* in soil samples collected from olive oil mill wastes evaporation ponds. The results showed a significant avoidance response (up to 40%) of the collembola, showing that the habitat function of the soil amended was compromised.

3.2.2.4. Reproduction assay with *Eisenia fetida*

The obtained results showed significant adverse effect on reproductive activity of *E. fetida* (Figure 15) was observed for S.CTL ($p < 0.05$), but no significant effects were recorded for the other treatments ($F_{13, 19} = 3.194$, $p = 0.059$). This effect may be related with the presence of calcium in the substrate from the nutrient solution used for irrigation (Hentati et al., 2015).

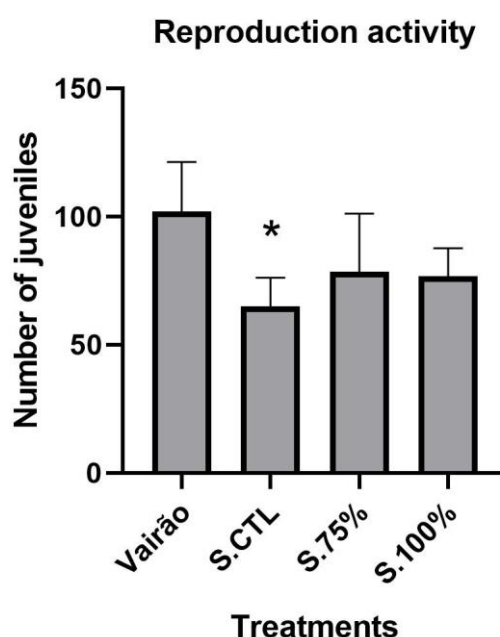


Figure 15 – Average values $\pm$ standard deviation of the reproductive activity of *E. fetida* by the end of 56-day exposure. * Indicates significant statistical differences from the control (Vairão soil), $p < 0.05$.

Campani et al. (2017) studied the toxicological effects of OOMW on *E. fetida*, assessing neurotoxicity, oxidative stress and genotoxicity after 72-hour exposure to different concentrations of raw OOMW (12.5%, 25% and 50%). The results showed that exposure to high doses of OOMW can cause the death of the earthworms and in low doses, adverse cellular and biochemical effects are observed, through neurotoxic effects, induction of oxidative stress and genotoxic effects. In another work with the aim to study the habitat function of soil samples collected from several evaporation ponds conducted by Hentati et al. (2016), both avoidance and reproduction assays

showed toxicity to *E. fetida*, with more than 60% avoidance and high inhibition of the reproductive activity ($EC_{20}=0.042\%$, $EC_{50}=0.138\%$), that may be related with the high phenol load of the wastes.

Therefore, taking in consideration the effect of the direct irrigation of OOMW or even soil amendments with olive oil wastes reported in the literature, it can be stated that the application of CW substrates to the soil after treatment of OOMW presents a more feasible and safer application in terms of toxicity to soil organisms. In fact, it can also be a valuable resource for plant growth, as observed in tomato dry biomass (for more details please see section 3.2.2.2.), due to the presence of nutrients that become adsorbed to the substrate.

4. Conclusion

The use and deposition of OOMW or olive oil mill wastes in soil have been reported by other authors to significantly affect and alter the physicochemical parameters of the soil. Moreover, several studies have also showed the negative effects and toxicity for several aquatic and soil organisms, allowing us to conclude that this is not a safe practice.

In this work, it was possible to observe a high efficiency of CW to treat OOMW and reduce its toxicity to biota, as well the safety of using the substrate of the CW as soil amendment.

An improvement in the physicochemical properties of the effluent was observed after its passage through CW through the increase of alkalinity and high removal rates of 99.17%, 98.10% and 78.71% for colour, COD, and Pt, respectively. The results also showed a decrease in the toxicity of the effluent, as in most cases no toxicity was recorded. Since the results were similar for both raw OOMW (E.100%) and diluted OOMW (E.75%), we can conclude that treatment of OOMW without dilution can be efficiently performed, reducing water waste. Thus, this work showed the potential applicability and efficiency of CW for the treatment of OOMW without the need for a pre-treatment, in order to improve physicochemical properties and reduce toxicity.

The assessment of the physicochemical properties of the soil amended with the CW substrates also showed that its application does not change considerably these properties, since no considerable changes in the soil properties were recorded. Furthermore, no toxicity/phytotoxicity was recorded for the substrates of the CW with OOMW. Thereby, it is possible to assume that the amendment of the soil with the CW substrates after its use to treat OOMW is a safe and viable solution, compared to the direct irrigation with OOMW. And in this way contribute to a circular and environmentally friendly economy, taking into account the seasonality of the OOMW production.

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6. Annexes

Table 1A – Hoagland's solution composition. Source: (Taiz & Zeiger, 2010)

	Compound	Conc of stock solution (g/L)	Vol for 1L of deionized water (mL)
1	KNO ₃	101.1	6
2	Ca(NO ₃) ₂ ·4H ₂ O	236.16	4
3	NH ₄ H ₂ PO ₄	115.08	2
4	MgSO ₄ ·7H ₂ O	246.48	1
5	KCl	1.864	2
	H ₃ BO ₃	0.773	
	MnSO ₄ ·H ₂ O	0.169	
	ZnSO ₄ ·7H ₂ O	0.288	
	CuSO ₄ ·5H ₂ O	0.062	
	Na ₂ MoO ₄ ·2H ₂ O	0.04	
6	FeEDTA	18.36	1

Table 2A – ASTM hard water solution composition. Source: (American Society for Testing and Materials, 1980; OECD, 2004; USEPA, 2002)

	Compound	Conc of stock solution (g/L)	Vol for 1 L of deionized water
Vitamins	CaSO ₄ ·2H ₂ O	1.2	0.12 g
	NaHCO ₃	19.2	10 mL
	MgSO ₄ ·7H ₂ O	24.57	10 mL
	KCl	0.8	10 mL
	B1	1500 mg/L	0.05 mL
	H	20 mg/L	
	B12	15 mg/L	

Table 3A – Woods Hole MBL solution composition. Source: (Stein, 1973)

		Compound	Conc of stock solution (g/L)	Vol for 1L of deionized water (mL)
Macronutrients	1	CaCl ₂ .H ₂ O	36.76	1
	2	MgSO ₄ .7H ₂ O	36.97	1
	3	NaHCO ₃	12.6	1
	4	K ₂ HPO ₄	8.71	1
	5	NaNO ₃	85.01	1
	6	Na ₂ SiO ₃ .9H ₂ O	28.42	1
Micronutrients	7	Na ₂ EDTA	4.36	1
	8	FeCl ₃ .6H ₂ O	3.15	1
	9	CuSO ₄ .5H ₂ O	0.001	1
	10	ZnSO ₄ .7H ₂ O	0.022	1
	11	CoCl ₂ .6H ₂ O	0.01	1
	12	MnCl ₂ .4H ₂ O	0.18	1
	13	Na ₂ MoO ₄ .2H ₂ O	0.006	1
Vitamins	14	Tris (pH 7.2)	200	2
		B1	0.1 mg/L	500 µL
		B7	0.5 mg/L	
		B12	0.5 mg/L	

Table 4A – Two-way and one-way ANOVA's summary (df, F ratio and p value) and post-hoc Tukey test results, regarding physicochemical parameters of the E.75% and E.100%. *RT – retention time; b.t. – before treatment

Two-way ANOVA							
	Endpoint	Source	df	F ratio	p value	Tukey test	Observed effects
OOMW	pH	RT	3	159.33	<0.001	b.t. ≠ 48h, 72h, week; week ≠ 48h, 72h	↗
		%OOMW	1	8.17	<0.001		
		RT*%OOMW	3	4.45	<0.001		
	EC	RT	3	188.93	<0.001	b.t. ≠ 48h, 72h, week; 48h ≠ 72h, week	↗
		%OOMW	1	44.75	<0.001	E.75% ≠ E.100%	
		RT*%OOMW	3	18.30	<0.001		
	Nt	RT	3	23.96	<0.001	48h ≠ b.t., 72h, week	↗

	%OOMW	1	2.03	0.173			
	RT*%OOMW	3	3.64	0.036			
COD	RT	3	542.83	<0.001	b.t. ≠ 48h, 72h, week	↘	
	%OOMW	1	1.55	0.231			
	RT*%OOMW	3	1.02	0.409			
Pt	RT	3	60.97	<0.001	b.t. ≠ 48h, 72h, week	↘	
	%OOMW	1	1.21	0.288	E.75% ≠ E.100%		
	RT*%OOMW	3	2.06	0.146			
Colour	RT	3	1744.70	<0.001	b.t. ≠ 48h, 72h, week	↘	
	%OOMW	1	43.63	<0.001	E.75% ≠ E.100%		
	RT*%OOMW	3	39.63	<0.001			
One-way ANOVA							
Endpoint	%OOMW	df1	df2	F ratio	p value	Tukey test	Observed effects
pH	E.75%	8	11	121.57	<0.001	b.t. ≠ 48h, 72h, week; week ≠ 48h, 72h	↗
	E.100%	8	11	52.17	<0.001	b.t. ≠ 48h, 72h, week; week ≠ 48h, 72h	
EC	E.75%	8	11	335.97	<0.001	b.t. ≠ 48h, 72h, week; 48h ≠ 72h, week	↗
	E.100%	8	11	65.62	<0.001	b.t. ≠ 48h, 72h, week; week ≠ 48h, 72h	
Nt	E.75%	8	11	30.41	<0.001	48h ≠ b.t., 72h, week	↗
	E.100%	8	11	4.08	0.050	b.t. ≠ 48h	
COD	E.75%	8	11	162.87	<0.001	b.t. ≠ 48h, 72h, week; week ≠ 48h, 72h	↘
	E.100%	8	11	622.23	<0.001	b.t. ≠ 48h, 72h, week; week ≠ 48h, 72h	
Pt	E.75%	8	11	31.65	<0.001	b.t. ≠ 48h, 72h, week; week ≠ 48h, 72h	↘
	E.100%	8	11	31.45	<0.001	b.t. ≠ 48h, 72h, week; week ≠ 48h, 72h	
Colour	E.75%	8	11	449.77	<0.001	b.t. ≠ 48h, 72h, week; week ≠ 48h, 72h	↘
	E.100%	8	11	1921.95	<0.001	b.t. ≠ 48h, 72h, week; week ≠ 48h, 72h	