Vertical sub-surface flow constructed wetland for olive oil mill wastewater treatment

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2019

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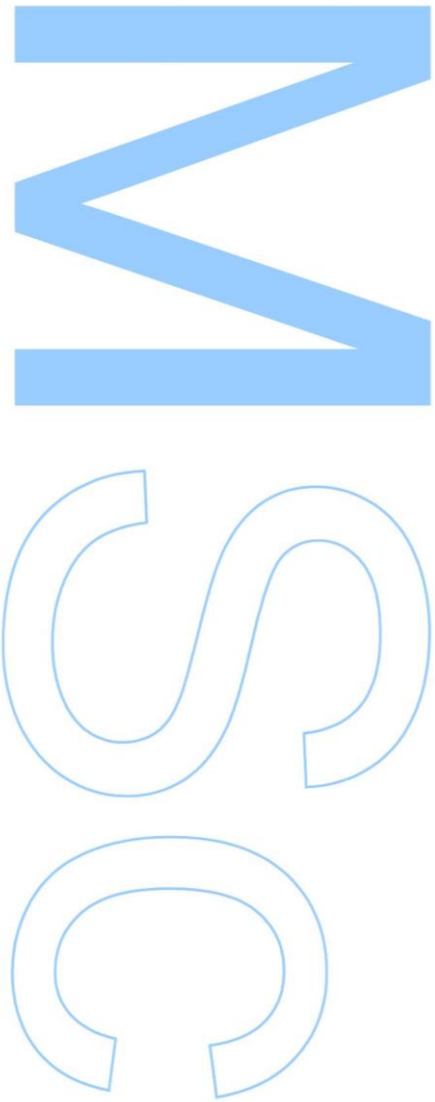

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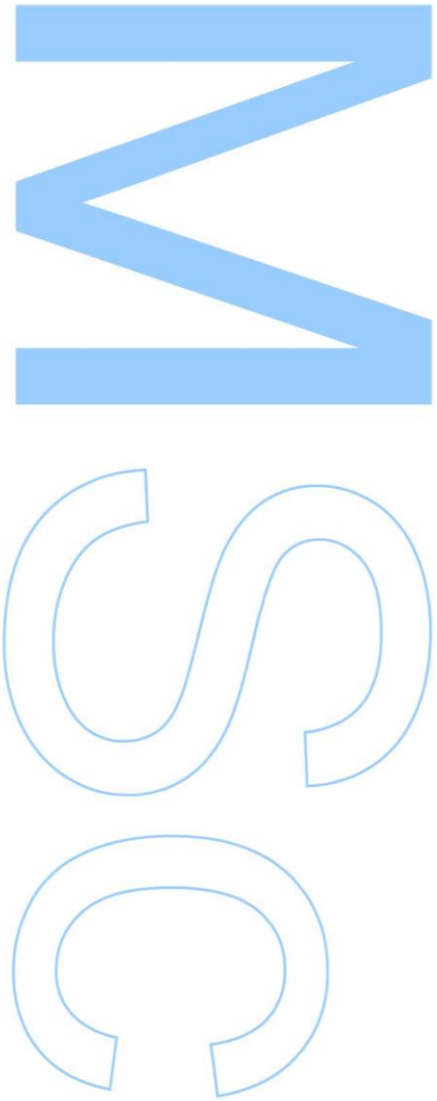

Agradecimentos

Quero começar por agradecer à Professora Doutora Ruth Pereira pela oportunidade que me deu de realizar esta dissertação e pela paciência e ajuda que forneceu no planeamento e no desenvolvimento da mesma. Um obrigado também à Doutora Cristina Almeida pelas ideias e pelos materiais que forneceu, que foram essenciais para a realização deste trabalho.

Quero também agradecer aos meus colegas do laboratório 1.39 pelo apoio que me deram ao longo deste tempo e por lidarem com a minha desorganização. Um obrigado especial à Verónica Inês Nogueira e ao Saul Simão por me terem guiado e ajudado em todos os passos desta etapa e pela paciência que tiveram com a minha falta de memória e distrações.

Um obrigado à Estibali Wilkie pela amizade, pela animação de cada encontro e por me dar alívio quando sentia que não ia conseguir terminar tudo a tempo. À Maria Pedrosa quero agradecer por ser uma excelente amiga e pelo interesse e disponibilidade que teve para ajudar.

Aos meus pais quero agradecer terem me dado todo o suporte que podiam até aqui e sempre me terem dado força para continuar os meus estudos. É graças a eles que consegui chegar até onde hoje estou.

O meu maior obrigado é ao Luís Marques, que esteve sempre ao meu lado e que me ajudou, sem hesitar, sempre que podia. Ele deu-me a força que precisava para continuar quando o desânimo e a ansiedade se tornavam demais para suportar. Sem ele nunca teria sido capaz de terminar esta dissertação.

Abstract

Constructed wetlands (CW) have been proposed for a more sustainable treatment of a variety of wastewaters while also being economic and easy to maintain. The treatment of olive oil mill wastewater (OOMW) with the use of a CW would be a green step in the production of olive oil. This dissertation aimed to assess the effectiveness of a pilot CW to improve the quality of OOMW in terms of its physico-chemical properties and toxicity. Furthermore, and considering the seasonality of this activity, the valorisation of the CW substrate at the end of the season, as a soil fertilizer, was also assessed.

Nine vertical sub-surface flow CWs (VSSCW) units were prepared with gravel, light expanded clay aggregates and artificial soil. *Thypa sp.* plants were planted and three treatments with three replicates each were tested: the control, which consisted of water with nutrient solution (E CTL), and OOMW effluent non-diluted (E 100%) and 50% diluted (E 50%). The effluent was recirculated and outflow samples were collected on three different experiments with different retention times. After three effluent load experiments the substrate was removed, grinded and stored for ecotoxicological tests with plants and soil invertebrates.

The pH of the effluent changed from 4.70 (±0.01) to 7.02 (±0.12) after CW treatment. Microtox toxicity test, *Raphidocelis subcapitata* growth inhibition test and Daphnia magna immobilization test showed a reduction of the toxicity of OOMW, after CW treatment. Avoidance tests with *Eisenia fetida* showed no avoidance of soils fertilized with CW substrate. A significant preference for the soils treated with substrate of the non-diluted OOMW CW replicates was recorded (P=0.0124). The substrate of the CW units was not phytotoxic to *Avena sativa* or *Lactuca sativ*a however they did not improve soil fertility.

Keywords

Constructed wetlands; olive oil mill wastewater; biological treatment; ecotoxicological assays; Vertical sub-surface flow; substrate valorisation

Resumo

As fito-ETAR têm sido propostas como um tratamento sustentável para uma variedade de efluentes por estas representarem um baixo nível de investimento e necessitarem de pouca manutenção. O tratamento de efluente de lagar de azeite (EFLA) com recurso a fito-ETAR pode-se tornar um passo importante na produção de azeite. Esta dissertação teve como objetivo perceber a eficácia de uma fito-ETAR piloto para melhorar a qualidade de EFLA em termos de propriedades químicas, físicas e toxicológicas. Além disso, considerando a sazonalidade da atividade da produção de azeite, a possibilidade da valorização do substrato da fito-ETAR, no final de cada estação de produção, como fertilizante do solo foi também avaliada.

Nove unidades de uma fito-ETAR de fluxo vertical piloto foram construídas com cascalho, argila expandida e solo artificial. Três tratamentos com três réplicas cada foram testados: controlo (constituído por água com solução nutritiva), EFLA não diluído (EFLA 100%) e o EFLA diluído a 50% (EFLA 50%). Plantas do género *Thypa* sp. foram colocadas em cada unidade. O efluente foi recirculado e as amostras foram recolhidas após três fases experimentais de tratamento na fito-ETAR. Após três períodos de tratamento o substrato foi removido, esmagado e armazenado para testes ecotoxicológicos com plantas e invertebrados de solo.

O pH do efluente aumentou de 4.70 (±0.01) para 7.02 (±0.12) após o tratamento. Tanto o ensaio de Microtox, como o ensaio de inibição de crescimento com a microalga *Raphidocelis subcapitata* e o ensaio de imobilização com *Daphnia magna* demonstraram reduções na toxicidade do efluente. Testes de evitamento com *Eisenia fetida* não revelaram evitamento dos solos com substratos da fito-ETAR incorporados. Uma preferência significativa foi encontrada pelo substrato das CW que receberam EFLA não diluído (P=0.0124). O substrato não foi fito-toxico para *Avena sativa* ou *Lactuca sativa* mas também não melhorou a fertilidade do solo.

Palavras-chave

Zonas húmidas construídas; Águas residuais da indústria do azeite; Tratamento biológico; Ensaios ecotoxicológicos; Valorização de substrato

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List of Abbreviations

COD - chemical oxygen demand

BOD - biochemical oxygen demand

FOG - fat, oil and grease

OOMW – olive oil mil wastewater

EU – European Union

WFD – Water framework directive

2P – two-phase continuous process

3P – three-phase continuous process

TPOMW - two-phase olive-mill waste

CW – Constructed wetland

SFCW – surface flow constructed wetland

SSFCW – sub-surface flow constructed wetland

VSSCW – vertical sub-surface flow constructed wetland

Chapter I - Introduction

1 Wastewaters: their source, specific characteristics and regulation

Regulations for the discharge of industrial wastewaters have been put in place to reduce the impacts of these externalities of human activities on receptor resources. The diversity of wastewaters in terms of their volume, physical and chemical properties and production regimes, can be a problem when treatment solutions and facilities have to be set up, since there is not a universal solution to all of them.

The food industry provides some examples of the variety of characteristics that wastewaters may present. The dairy industry wastewater, for example, has high quantities of biochemical oxygen demand (BOD), resulting from milk components such as proteins, fat, lactose and lactate. These wastewaters also have high sodium content due to the usage of caustic soda for cleaning processes, as well as a myriad of detergents and sanitizers. The production is intermittent thorough the day and linked with different processing stages (Hwang and Hansen, 1998; Demirel, Yenigun and Onay, 2005; Munavalli and Saler, 2009; Raghunath *et al.*, 2016). The fish canning industry produces wastewaters with high BOD and suspended solids rich in proteins and lipids. The fish processing results in a wastewater which contains great quantities of ammonium and FOG (fat, oil and grease). High phosphorous levels are also a characteristic, both from fish and cleaning products. The wastewaters produced by this industry can have very different characteristics depending on the processing stage where they are generated, the type of fish, the type of processing and additives used (Chowdhury, Viraraghavan and Srinivasan, 2010; Cristóvão *et al.*, 2016). The olive oil industry, on its turn, produces wastewaters that can have different characteristics depending on a variety of factors such as the storage time, the type and maturity of the olive and the extraction method. Overall olive oil mill wastewater (OOMW) has a dark color and foul smell, tends to be acidic, has high organic content, with low BOD/COD ratios indicating a low biodegradability and a high concentration of phytotoxic phenolic compounds (Azbar *et al.*, 2004; Niaounakis and Halvadakis, 2006; Aggoun *et al.*, 2016; Gikas, Tsakmakis and Tsihrintzis, 2018).

In 2000 the European Union (EU) put into place the directive 2000/60/EC which is known as the EU Water Framework Directive (WFD). In Portugal the directive was transposed for the national legislation by the law n. º 58/2005, of 29 of December. The WFD had the objective to either maintain or achieve a good ecological and chemical status of the water resources in the EU countries. After this directive other daughter regulations and directives were launched. On 24 November of 2010, the directive 2010/75/EU for industrial emissions was published. This directive regulated industry emissions by obliging

a series of specified industries to apply for a certificate of operation, which has to take into account limit values for emissions, monitoring programs and protection measures for the soil and groundwaters. In these guidelines, OOMW were not specifically addressed.

2 The particular case of olive oil industry wastewater

In many Mediterranean countries, including Portugal, the olive oil industry is a very important economic activity being responsible for the production of great volumes of a very hazardous wastewater, that still represents a great environmental problem in many regions (Paraskeva and Diamadopoulos, 2006; Kapellakis, Tsagarakis and Crowther, 2008; Koutsos, Chatzistathis and Balampekou, 2018). Therefore effective and few expensive treatment methodologies to deal with these effluents, are required to achieve the targets of water protection, without compromising the sustainability of a very important economic activity.

Olive oil is mainly produced in the Mediterranean countries, with countries within the European Union (EU) representing around 70% of the world production in the 2016/2017 campaign. As it can be seen in table 1, between 2009/2010 and 2016/2017, Portugal, in particular, produced on average 74 000 tons of olive oil per campaign, representing around 3.8% of all olive oil produced in the EU in this last period, with a forecasted increase to 115 000 tons in 2019 (Internacional Olive Council, 2019).

Table 1: Olive oil production in Portugal per campaign from 2009 to 2017 (Internacional Olive Council, 2019).

Olive oil production in Portugal	
Campaign	Production in tons
2009/2010	**63000**
2010/2011	**63000**
2011/2012	**76000**
2012/2013	**59000**
2013/2014	**92000**
2014/2015	**61000**
2015/2016	**109000**
2016/2017	**69000**

With the increase in olive oil production, the management of olive oil wastewater (OOMW) becomes even more concerning.

There are three main olive oil extraction methods, the press process, which is the most traditional, and the continuous centrifugation process, this one includes the 3-phase (3P) and the 2-phase (2P) processes, being the later the most recent approach. Figure 1 is a schematic representation of these different production processes.

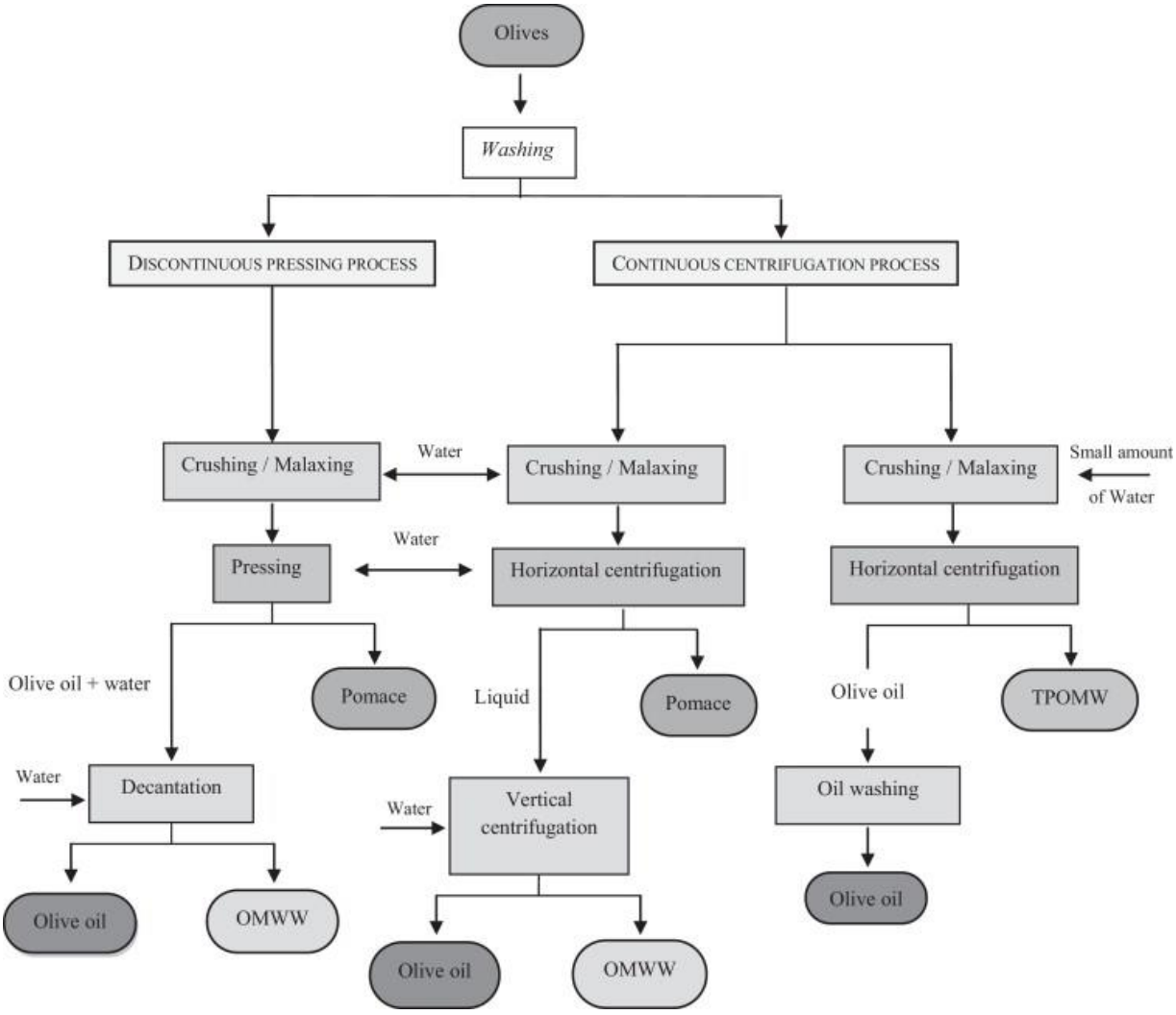

Figure 1: Schematic representation of olive oil production processes. OMWW - olive mill wastewater. TPOMW - two-phase olive mill waster (Dermeche *et al.*, 2013).

In the press method, after being washed the olives are crushed and the resulting olive paste is then pressed, leaving olive pomace (a mix of olive pulp, peel, stone and water) and a mixture of oil and water is obtained. Decantation follows, and the olive oil is separated giving rise to a wastewater. Olive oil, pomace and OOMW are the end products

of this process. This method requires less capital to build facilities but needs more workforce. It also produces less wastewater, although more concentrated than in the 3P method (Vlyssides *et al.*, 1998; Azbar *et al.*, 2004; Dermeche *et al.*, 2013; Domingues *et al., in press*).

In the 3P method, after being crushed, the paste goes through horizontal centrifugation, where pomace is separated from the liquid phase. Vertical centrifugation of the liquid phase follows, allowing the separation of olive oil and the OOMW. In the 2P method there is no separation of solid and liquid phase wastes. After horizontal centrifugation occurs, a pomace with high water content, also known as two-phase olive-mill waste (TPOMW) is obtained as waste. The 3P method requires more water, which results in higher volumes of wastewater produced. Both continuous centrifugation methods have a better production of olive oil and better control of the process and require less space than the traditional method. The 2P method despite having no water usage after the washing process, produces TPOMW that is hard to manage (Azbar *et al.*, 2004; Dermeche *et al.*, 2013; Domingues *et al., in press*).

Overall the wastewater formed during the production of the olive oil has a dark colour, foul smell, acidic to neutral pH (3 up to 7), high organic content (COD values of 15g/L to 390 g/L), with low BOD/COD ratios indicating a low biodegradability, high concentration of phenolic compounds (5g/L up to 80 g/L), high conductivity caused by the presence of dissolved salts like potassium, calcium, magnesium and sodium, high concentration of phosphate and nitrogen, high quantities of total solids, oil and grease (Azbar *et al.*, 2004; Niaounakis and Halvadakis, 2006; Gikas, Tsakmakis and Tsihrintzis, 2018).

The discharge of OOMW into natural water bodies causes severe damages on aquatic communities due to the high organic load and nutrients discharged, that cause oxygen depletion and eutrophication; the development of grease films on the water surface that block light and gases exchange; the discoloration of water because of dark coloured polyphenols resulting from oxidation and polymerization of tannins, and the enhanced toxicity due to the anti-microbial and phytotoxic properties of organic acids and phenolic compounds (Paraskeva and Diamadopoulos, 2006; Kapellakis, Tsagarakis and Crowther, 2008; Koutsos, Chatzistathis and Balampekou, 2018).

Besides the high toxicity of OOMW, the production of olive oil is limited to a short period of time, around three to four months and olive oil mills tend to be scattered. Also the production tends to be made by small and medium scale installations that do not have the necessary capital to make high investment in treatment plants (Paraskeva and

Diamadopoulos, 2006; Özgün *et al.*, 2016; Koutsos, Chatzistathis and Balampekou, 2018).

In Portugal there is no specific regulations for the treatment of OOMW. In 2000, the joint dispatch nº 118/2000, of 2 of February, from the Ministry of Agriculture, Rural Development and Fishing, and the Ministry of Environment and Territory Planning, was published and promoted the modernization of the olive oil industry by awarding licences only to mills that could implement facilities or processes aimed in reducing their environmental impact. Also, in this year, another joint dispatch from the same Ministry of Economy and the Ministry of Agriculture, Rural Development and Fishing, n. º 626/2000 of 6 of June, was published regulating the spread of OOMW on agricultural soils. This dispatch limited the volume of wastewater that can be spread per hectare, the type of cultures that could be irrigated and limitations on the usage near water resources, populations and protected areas. Also, this dispatch ordered the pre-treatment of the OOMW to correct its pH, before being discharged on soils.

The traditional, less expensive, and less technology-dependent method of dealing with OOMW is the storage in evaporation ponds were the liquid phase is evaporated during the hottest months. This method has some social unacceptance and still has environmental risks due to the bad odour released, the possibly of leaking of OOMW to ground and surface waters, the occupation of a great area of soil with ponds, if an effective evaporation surface is to be obtained and the production of another waste, which is a dark, foul smelling and difficult to remove sludge highly concentrated in polyphenols (Hachicha *et al.*, 2009; Herouvim *et al.*, 2011; Kapellakis *et al.*, 2012).Although, this latter waste is being managed to produce fireplace pellets.

The valorisation of OOMW for irrigation is also a common practice. By having high quantities of nutrients and organic matter, OOMW could be used as an agricultural fertilizer. However, given its questionable quality as fertilizer, the application of OOMW on some types of soil and without well-established guidelines, can have short and long-term negative impacts on soil quality, but also on crops production (Muscolo *et al.*, 2010; Asfi, Ouzounidou and Moustakas, 2012; Massoudinejad, Arman and Aghayani, 2014; Ayoub *et al.*, 2016).

As said before, the toxicity of OOMW, along with its production seasonality, management difficulties and the low medium scale of investment possibilities are all difficulties that challenge the finding of an adequate treatment for OOMW. In the context of many Mediterranean countries, the use of constructed wetlands can be a possibility, thanks to their potential efficacy for dealing with OOMW, low-implementation costs, their perfect integration in rural areas and potential to contribute to a circular economy.

3 Constructed wetlands

Wetlands are natural structures that form on the transition zone between terrestrial and aquatic ecosystems and are deeply connected to changes in water level. They are habitat for many unique organisms from plants, animals, fungi, bacteria and others that are adapted to changes in water and oxygen availability (Keddy, 2010). Wetlands are known for their performance of a variety of ecosystem services. These ecosystems have an important role in water quality thanks to their impact in the biochemical cycles of nitrogen, carbon and sulphur (Jenkins *et al.*, 2010; Mitsch, Bernal and Hernandez, 2015).

The ability of wetlands to remove organic pollutants that are common in most wastewaters produced by human activities lead to the construction of artificial constructed wetlands (CW). CW appear as a green technology, with low-cost of implementation and maintenance, that can be used as a substitute for the traditional wastewater treatment plants, especially in areas where wastewater production has low volumes or is produced seasonally, the latest being the case of olive oil wastewater (Hammer and Bastian, 1989; Wu *et al.*, 2015; Ilyas and Masih, 2017; Machado *et al.*, 2017; Koutsos, Chatzistathis and Balampekou, 2018).

There are many types of CW, that may differ on many aspects from the type of plants and substrate used, to the wastewater treated, how often it is loaded and the residence time of the wastewater, all these parameters can modulate the effectiveness of the treatment (Li *et al.*, 2008; Wu *et al.*, 2015; Stanković, 2017).

Usually CW can be classified based on the flow of the wastewater through it (Ghermandi, Bixio and Thoeye, 2007). Figure 2 shows a schematic representation of the flow patterns of the main types of CW.

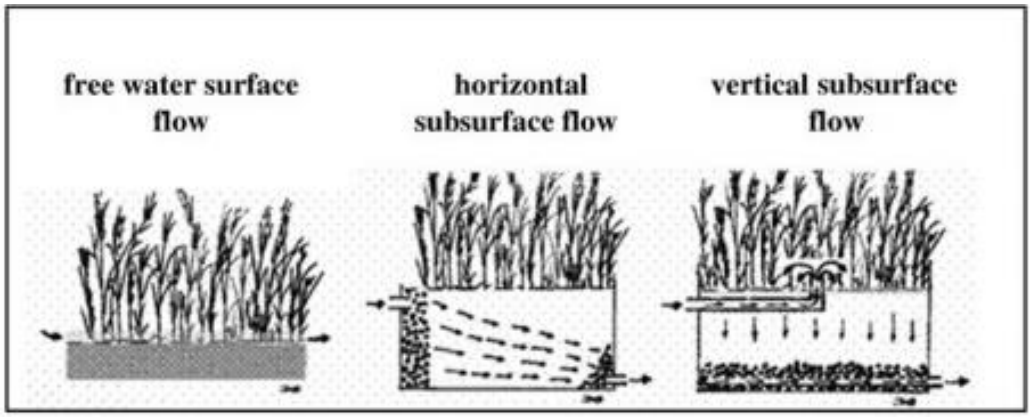

Figure 2: Different types of flow in constructed wetlands (Ghermandi, Bixio and Thoeye, 2007).

In a Surface Flow CW (SFCW) the wastewater flows through the surface of the substrate, in contact with the plants and air, until its removal. SFCWs are cheaper than Sub-surface Flow CWs (SSFCW), can treat wastewater with high amounts of suspended solids, without clogging and, are able to have both anoxic and aerobic zones, depending on the wastewater depth, which contributes for a great efficiency in nitrogen removal. On the other hand, in SFCWs the water has a limited contact with the substrate causing reduced treatment efficiency. Other disadvantages include foul smell and attraction of insects thanks to requirement of a wide surface area to be as effective as SSFCW (Halverson, 2004; Vymazal, 2008; Valipour and Ahn, 2016).

SSFCWs can be divided in two types, horizontal and vertical. In horizontal SSFCW the wastewater always flows horizontally under the surface of the substrate, while on vertical SSFCWs (VSSCW) the wastewater is loaded on the surface of the substrate and percolates through it, under gravity, being removed in the bottom. Horizontal SSFCWs are effective in the removal of suspended solids but tend to have low availability of oxygen, reducing their capability for denitrification, while on VSSCWs the air trapped between injections of wastewater contributes for the oxygenation of the substrate and subsequently better removal of nitrogen. Both have a higher efficiency in removing pollutants then a SFCW thanks to the substrate matrix that allows the development of a biofilm. SSFCWs tend to suffer clogging of the substrate especially when wastewater with a high amount of solids are treated (Stefanakis, Akratos and Tsihrintzis, 2014; Wu *et al.*, 2015; Valipour and Ahn, 2016).

The substrate used in the CW has an important role in the effectiveness of the treatment. The substrate can act as an important mechanical treatment since the percolation of the wastewater through it leads to the filtration of pollutants. The adsorption

of substances such as phosphorous, ammonium and metals, is also another important role the substrate can have, also assisting the microbial community in the removal of biodegradable pollutants. The microbial community tends to form biofilms around the substrate, thus the higher the surface area is, the higher quantity of microbial growth is achieved. The traditional substrate used is composed by sand and gravel (Zamora *et al.*, 2019). Other man-made substrates, such as zeolite, expanded argyle or polyethylene plastic, with a high surface area and better adsorption capacities have also been used and can have better efficiencies than those traditionally used (Lu *et al.*, 2016; Yang *et al.*, 2018).

CWs can also be a good treatment approach for OOMW, given their low cost, the low area of soil required to improve their efficiency and the possibility to reuse their components. Such requirement can be fulfilled by the olive oil industry, with a low capital for investment, as is the case of small mills that exist in rural areas, where soil is also frequently available for their establishment.

4 Dissertation objective and structure

In this dissertation a pilot VSSCW was tested to treat and reduce the toxicity of an OOMW from a small mill in the Centre of Portugal (Viseu, Portugal).

This work had two major objectives, namely: i)To understand the ability of a vertical VSSCW pilot to treat OOMW improving their physical and chemical properties and reducing its toxicity and, ii) to test the possible re-use of the wetland substrate, at the end of the production season, as a soil fertilizer.

Chapter II – OOMW toxicity removal in a pilot constructed wetland

1 Introduction

Olive oil mill wastewater (OOMW) has a high toxicity. This wastewater is known to have a dark colour, foul smell, acidic pH, high organic content with low BOD/COD ratios, indicating a low biodegradability and high concentration of phenolic compounds (5g/L up to 80 g/L). (Azbar *et al.*, 2004; Niaounakis and Halvadakis, 2006; Gikas, Tsakmakis and Tsihrintzis, 2018). The discharge of OOMW into natural water bodies causes severe damages on aquatic communities due to the high organic load and nutrient contents of the discharges; the development of grease films on the water surface that block light and gases exchange; the discoloration of water and, the enhanced toxicity due to the anti-microbial and phytotoxic properties of some major components, such as phenolic compounds (Paraskeva and Diamadopoulos, 2006; Kapellakis, Tsagarakis and Crowther, 2008; Koutsos, Chatzistathis and Balampekou, 2018).

Besides the high toxicity of OOMW, the production of olive oil is limited to a short period of time, around three to four months and olive oil mills tend to be scattered. Also, the production tends to be made by small and medium scale installations that do not have the necessary capital to make high investment in treatment plants (Paraskeva and Diamadopoulos, 2006; Özgün et al., 2016; Koutsos, Chatzistathis and Balampekou, 2018).

Constructed wetlands (CW) are a green technology, with low-cost of implementation and maintenance and which can be a solution in areas where wastewater production has low volumes or is produced seasonally, the latest being the case of olive oil wastewater (Hammer and Bastian, 1989; Wu *et al.*, 2015; Ilyas and Masih, 2017; Machado *et al.*, 2017; Koutsos, Chatzistathis and Balampekou, 2018). CWs can be classified based on the flow of the wastewater through it. Two major types can be distinguished: surface flow CW and sub-surface flow CW (SSFCW). On the first, wastewater flows across the surface, without infiltration on the substrate, while on SSFCW the wastewater always percolates through the substrate. SSFCW can also be divided in two groups: On horizontal SSFCW the wastewater is continually added on the substrate and flows horizontally, being removed on the other side of the CW. In the vertical SSFCW (VSSCW) the wastewater is loaded between intervals of time, on the surface of the substrate, and percolates through it under gravity, being removed in the bottom.

VSSCW were chosen to treat OOMW do to their advantages when compared with other CWs with different flow patterns. By loading wastewater on the top, it gives the possibility of aeration between effluent loads and keeps the medium aerobic allowing a better nitrogen removal, through denitrification. They also have a better organic matter removal capacity and they need less area to be as effective as the other CWs. On the other hand, these CWs are prone to clogging specially when treating effluents with high amounts of suspended soils (Stefanakis, Akratos and Tsihrintzis, 2014; Wu *et al.*, 2015; Valipour and Ahn, 2016).

A few studies applied VSSCWs to the treatment of OOMW (Yalcuk, Pakdil and Turan, 2010; Herouvim *et al.*, 2011; Dordio and Carvalho, 2013; Achak *et al.*, 2019). The usage of natural substrates, such as gravel and sand (Zamora *et al.*, 2019), were common to all studies found. While both, Yalcuk et al, (2010) and Dordio et al, (2013) used man made substrates, zeolite and LECA®, respectively (Yalcuk, Pakdil and Turan, 2010; Herouvim *et al.*, 2011; Dordio and Carvalho, 2013; Achak *et al.*, 2019).

In all these studies the effectiveness of removal of most pollutants from the OOMW was high but the authors confirmed that CW treatment was not sufficient to comply with EU rules to wastewater discharge, thus more studies are needed to analyze the use of CW for a more sustainable olive oil production. In this work a pilot VSSCW was built using gravel, LECA® and OECD soil as substrate. *Typha latifolia* was the plant species selected to be implemented on the CW for their availability as well as their ability to withstand conditions of anoxia, changing water availability and high organic matter contents (Vymazal, 2011). The treated effluent then run through a series of ecotoxicological assays with standard aquatic species to evaluate the ability of the pilot CW to decrease the toxicity of the OOMW.

In this work it was hypothesized that the combination of this substrate and this species from flooded areas such as brackish marshes in a VSSCW would be able to reduce the toxicity of OOMW to aquatic organisms.

2 Material e methods

2.1 Pilot constructed wetland

Nine pilot CW units were built on plastic boxes (28 cm Height; 24 cm Wight; 34 cm Length) with a tap on the bottom to collect the OOMW after it has percolated by gravity through the substrate (Figure.1). On the bottom of the boxes a collector tube with holes was connected to the tap to equally remove the wastewater from all the area of the bottom, preventing

accumulation in some parts. The substrate was added in different layers (Fig. 1): gravel was the first, to prevent clogging of the collector tube. Commercial LECA® was the second layer of substrate. This material has high surface area/volume ratio allowing the development of biofilms and it is capable of adsorbing organic molecules (Dordio *et al.*, 2007; Dordio and Carvalho, 2013). OCDE artificial soil was added on the top, as a third layer for plant support. This soil was composed by 70% of sand, 20% of kaolin clay and 10% *of Sphagnum* peat (OECD, 1984). The height of each substrate was 4 cm, 2 cm and 2 cm respectively.

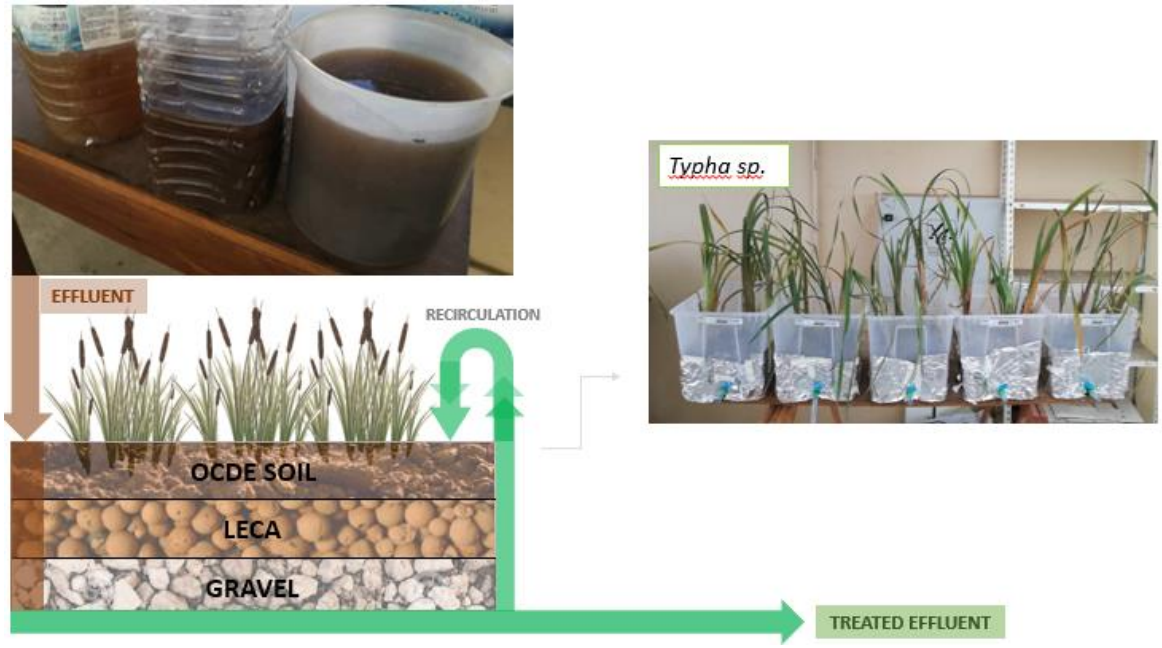

Figure 1 – Pilot constructed wetland and corresponding substrate composition and plants

Typha latifolia plants were collected at Salreu (Ria de Aveiro, Centre of Portugal) (figure 2) and maintained in buckets with water from the marshland, where they were collected, before being planted in the CW boxes. The plants were maintained at room temperature and natural photoperiod.

Figure 2. Geographical location of the point of *Typha latifolia* plants collection (Goggle Maps).

In each CW unit/box 5 plants with more or less the same height were planted and the system was left to stabilize for 4 weeks. During the first week the boxes were irrigated with diluted marsh water with 1:2 of tap water, after this week and for 3 weeks a nutritive solution (Hogland solution: see table 1A of the attachments), consisting of 14 ml of nutrients per 1 L of deionized water, was used. To each unit, 1L of the nutrient solution was added two times per week to keep the substrate humidity. Before new water additions taps were opened to remove the excess of previously added water for recirculation and aeration of substrate.

2.2 Experimental design

Three treatments with 3 replicates each were prepared. The control (E CTL) which only received tap water with nutritive solution and the units that received the non-diluted OOMW (E 100%) and the 50% diluted OOMW (dilution with tap water) (E 50%).

The OOMW treatment of the CW units was performed in three experiments:

Experiment 1: In this experiment the OOMW was treated in the CW systems for one week. 1 L of the corresponding effluent dilution or nutrient solution was added to the top of the substrate in each replicate. Recirculation of the effluent was made every two days and samples of 5 ml were collect (Sample times: 1.1; 1.2; 1.3) on each recirculation day from all the replicates, in a total of 27 samples. By recirculation we want to describe the removal of the effluent in the bottom of each box and the re-load on the surface of the substrate. This was done to prevent the formation of anoxic zones in the CW system, which could affect treatment. The non-treated OOMW was also stored for analysis. At the end of the week all the effluent in the boxes was discarded. These samples were frozen at -20ºC and later were used for Microtox assays.

Experiment 2: In this experiment the OOMW was treated in the CW systems for two weeks. For that, 1.5 L of the respective effluent dilution or nutrient solution was added to each replicate. The recirculation of the effluent was made at each 3 days. Samples of 50 mL were collected from all the replicates in each recirculation day and at the end of the two weeks (Sample times: 2.2; 2.3; 2.4; 2.5) from each replicate, in a total of 36 samples. Also, when the effluent collected on the recirculation was lower than 1L, OOMW was added until 1.5L were reached again. All samples were frozen at -20 ºC. The collected effluent in the end two weeks was also used for assays with *Raphidocelis subcapitata* and *Daphnia magna*.

Experiment 3: In this experiment the OOMW was treated in the CW systems also for two weeks with a with recirculation system and collection of 50ml samples every 3 days (Sample times: 3.1; 3.2; 3.3; 3.4; 3.5), leading to 45 samples. The initial volume of effluent added to the CW units was 1.5L. At the end of the first week during the recirculation more OOMW was added to the collected effluent to perform the initial volume of 1.5L. All the effluent was collected on the last day. All samples were frozen at -20 ºC. The final effluent samples were also used for assays with *Raphidocelis subcapitata* and *Daphnia magna*.

A sample of raw OOMW was also obtained which was used in assays of Microtox® and assays with *Raphidocelis subcapitata.*

The pH of all the sample of experience 2 and 3, as well as, OOMW, were measured using a Hanna Edge multiparameter with an HI 11310 pH electrode while the conductivity was measured using an HI 763100.

2.3 *Daphnia magna* and *Raphidocelis subcapitata* culture conditions

D. magna neonates were obtained from laboratorial cultures kept under environmental controlled conditions of temperature (21±1ºC) and photoperiod (16h^{L}:8h^{D}). The cladocerans were cultured in 1L glass pots in ASTM medium (ASTM, 2006). Only neonates, with less than 24h, from the 3rd to the 5th broods were used for the acute assays.

Raphidocelis subcapitata microalgae was obtained from a laboratorial culture maintained at LABRISK (FCUP). Three days before the assays, around 5ml of Woods Hole MBL medium with an inoculum of *R. subcapitata* were added to a sterilized Erlenmeyer with MBL medium. The flask was kept at continuous conditions of temperature (21±1ºC) and photoperiod (16 h^{L}:8 h^{D}), to achieve exponential growth before the assay.

2.4 Ecotoxicological assays

2.4.1 Bioluminescence inhibition assay with *Allivibrio fischeri* (Microtox® assay)

The Microtox® assay was performed using a Microtox® Analyser Model 500, which incubates the samples and the bacteria and registers the bacteria luminescence.

In this assay the bacteria are expose to different dilutions of each sample and the light emission is registered, both before (I0) and after 5, 15 and 30 minutes of bacteria exposure to the sample (If). The difference between the initial and final light emission is attributed to the effect of the sample.

A. fischeri delivered lyophilized by the manufacture was the bacteria used in the test. It was rehydrated with a reconstitution solution right before starting the assay.

The EC_x values were calculated using the software MicrotoxOmni (Azur Environmental, 1998).

2.4.2 *Raphidocelis subcapitata* growth inhibition test

This assay followed the protocol OECD 201 (OECD, 2011) to determine the effect of the treated effluent samples collected on the second and third experimental steps on the growth of the freshwater algae *Raphidocelis subcapitata*. Before the assay, the number of cells in the inoculum (see section 2.3) was counted using a Neubauer chamber to determine the algae concentration on the inoculum. The inoculum was then diluted with Woods Hole MBL medium for a density of 10^5 cells/ml.

Three composed samples were tested (E CTL; E 100%; E 50%) by mixing the replicates of the effluent samples 2.5 and 3.5 collected for each CW pilot treatment. Six dilutions (100-13.2%) obtained with a factor of 1.5 were tested. The sterilized microplates were loaded, under sterilized conditions, according to the scheme represented in figure 3.

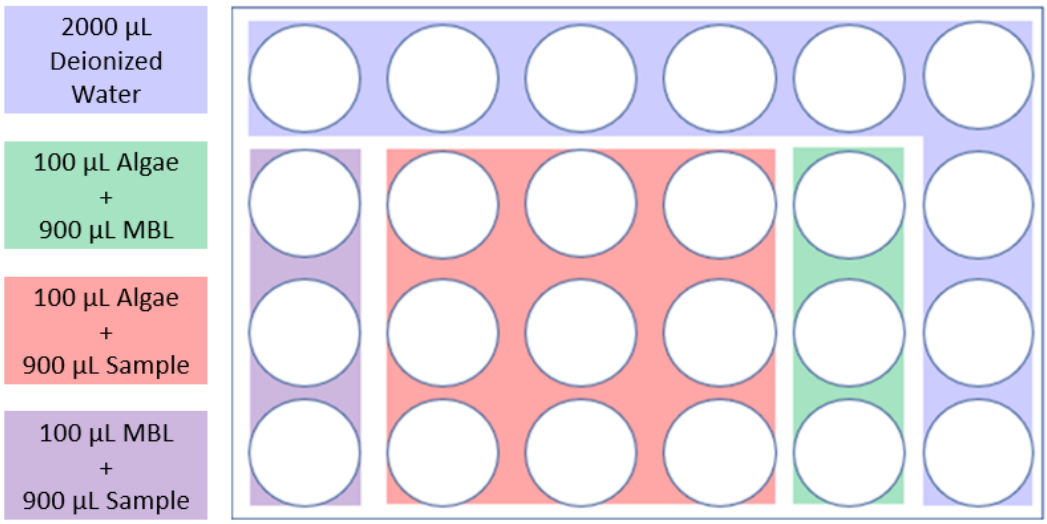

Figure 3 – Schematic representation of the strategy followed to add the sample dilutions to each test microplate in the assay with *R. Subcapitata*

The plates were placed on an orbital shaker under environmental controlled conditions of temperature (21±1ºC) and photoperiod (16 h^L:8 h^D), for 72h. At the end of the test, the absorbance of the content of each well was measured at 440 nm. The absorbance of each replicate was used to calculate cell's density, using the following equation:

Concentration (Cell/mL) = (Abs-0.0025)/0.00000005

The average algae growth rate was calculated using the equation:

$$\mu_{i-j} = \frac{\ln X_j - \ln X_i}{t_j - t_i} \ (\text{day}^{-1})$$

In which:

µi-j – average growth rate from time i to j

Xi - biomass in time i

Xj - biomass in time j

The percentage of inhibition of the growth rate was also calculated for each replicate of each sample dilution, using the following equation:

$$\%I_r = \frac{\mu_C - \mu_T}{\mu_C} \times 100$$

In which:

%Ir – percentage of inhibition of the average specific growth rate
μC – medium of the average growth rate for the control group
μT - medium of the average growth rate for each replicate

The Statistica® software was used to estimate EC_{10}, EC_{20} and EC_{50} values and their 95% confidence intervals for each tested sample (i.e. composite samples collected from each CW pilot treatment - 2.5 and 3.5), after adjustment of non-linear regression to the data.

2.4.3 *Daphnia sp.* acute immobilisation test

This assay followed the standard protocol No. 202 from OECD for *Daphnia* sp. acute immobilization test (OECD, 2004). Composite samples of the effluent collected at end of the third experiment (3.5 - E CTL; E 100%; E 50%) were tested. Six dilutions of each sample were prepared with ASTM medium, from 100% 13.2%. A control with ASTM medium was also tested.

For each previously described dilution and for the control of the test, four test replicates in glass tubes with 25 mL and 5 neonates of *D. magna* were prepared. The tubes were kept under controlled conditions of temperature (21±1ºC) and photoperiod (16 h^L:8 h^D). The duration of the assay was 48h and the number of immobilized organisms in each test tube was recorded at 24 and 48 hours of exposure.

3 Results and discussion

3.1 pH and conductivity

For both experiments 2 and 3, the pH of the effluent became close to neutrality after passing through the CW, and this was not affected by the continuous addition of more OOMW. These results showed in table 1 are in accordance with the literature, and may have been caused by the pH-buffering capacity of the substrate, as well as due to oxidation or dilution of carbonate salts present in substrate, such as soil which had a pH of an approximated 6 (Herouvim *et al.*, 2011; Dordio and Carvalho, 2013; Achak *et al.*, 2019).

Table 1: pH values (mean and standard deviation, n=3) for each sample collected on the second and third experiments. i represents the initial pH of the OOMW sample, diluted and non-diluted the numbers represent the samples collected in the CW units in each period of the second and third experiments.

			i	2.2		2.3	2.4		2.5
Treatm.	2	E 100%	4.7 ± 0.0	7.0 ± 0.0	OOMW added	7.1 ± 0.1	7.0 ± 0.1	OOMW added	6.9 ± 0.1
		E 50%	4.8 ± 0.0	7.2 ± 0.0		7.2 ± 0.0	7.2 ± 0,0		7.0 ± 0.0
		E CTL	-	7.3 ± 0.1		7.5 ± 0.2	7.2 ± 0.1		7.1 ± 0.1

			i	3.1	3.2		3.3	3.4	3.5
Treatm.	3	E 100%	4.7 ± 0.0	6.9 ± 0.1	7.0 ± 0.1	OOMW added	7.5 ± 0.5	7.0 ± 0.1	6.9 ± 0.1
		E 50%	4.8 ± 0.0	7.0 ± 0.1	7.3 ± 0.1		7.3 ± 0.4	7.0 ± 0.0	7.1 ± 0.1
		E CTL	-	7.1 ± 0.1	7.1 ± 0.1		7.5 ± 0.4	7.0 ± 0.1	7.3 ± 0.3

After passing through the CW the conductivity of the treated OOMW increased in all treatments compared to the raw OOMW, as seen in table 2. Yalcuk *et al*, (2010) found a similar occurrence and identified the release of ions from the substrate as the cause, and in fact this can be confirmed with our E CTL samples from the CTL units of the CW, where only tap water was added. For these samples the conductivity recorded was also high. Other explanation for the increased conductivity could be the mineralization of the organic matter (Achak *et al.*, 2019). This enhanced conductivity persisted throughout experiment 2 and 3, suggesting that the wash of the CW substrate with the effluent was not sufficient to overcome this problem.

Table 2: Conductivity values in mS/cm (mean and standard deviation, n=3), for each sample collected on the second and third experiments. i represents the initial pH of the OOMW sample, diluted and non-diluted the numbers represent the samples collected in the CW units in each period of the second and third experiments.

			i	2.2		2.3	2.4		2.5
Treatm.	2	E 100%	1422 ± 8	2255 ± 301	OOMW added	2295 ± 251	2351 ± 240	OOMW added	2258 ± 221
		E 50%	766 ± 57	1806 ± 33		1758 ± 73	1843 ± 72		1761 ± 108
		E CTL	-	1414 ± 234		1324 ± 207	1407 ± 217		1250 ± 166

			i	3.1	3.2		3.3	3.4	3.5
Treatm.	3	E 100%	1422 ± 8	2221 ± 162	2343 ± 128	OOMW added	2421 ± 126	2625 ± 220	2640 ± 203
		E 50%	766 ± 57	1460 ± 44	1548 ± 105		1511 ± 131	1633 ± 144	1794 ± 113
		E CTL	-	851 ± 52	916 ± 68		832 ± 29	812 ± 123	865 ± 57

3.2 Bioluminescence inhibition assay with *Allivibrio fischeri* (Microtox® assay)

Raw OOMW was highly toxic to *A. fischeri* causing an EC_{50} of 2.15% at 30 minutes of exposure. These results are in accordance with the literature. Babíc et al, (2019) found EC_{50} values 0.24% for raw OOMW, while Paixão et al, (1999) reported EC_{50} values ranging from 0.16 to 1.24% for different OOMWs. Also, Amaral et al, (2012) reported inhibitions of 100% of the luminescence when these bacteria were exposed to raw wastewater. Considering the EC_{50} values recorded for the non-diluted sample a reduction of about 9X was observed from the first to the third passage of the OOMW effluent on the CW.

Table 3: EC_{20} and EC_{50} values and corresponding 95% confidence intervals, recorded after 30 minutes of exposure to the bacteria *A. fischeri*, in the Microtox assay for a replicate of each sampling 1.1, 1.2, 1.3 and raw OOMW. When it was not possible to estimate an EC value, the highest effect percentage (H.E.) was recorded. Only the replicate were it was possible to calculate EC_{20} and EC_{50} are shown.

		EC20 %	EC50 %	H.E. %
	OOMW	0.37 (0.17 - 0.57)	2.15 (1.16 - 3.15)	-
1.1	E 100% R3	4.95 (4.00 - 5.90)	20.98 (18.67 - 23.28)	-
	E 50% R1	11.72 (9.34 - 14.09)	35.10 (31.12 - 39.07)	-
	E CTL R2	-	-	9.954
1.2	E 100% R3	8.52 (5.85 - 11.18)	19.41 (14.25 - 24.56)	-
	E 50% R1	-	-	34.63
	E CTL R2	-	-	13.1
1.3	E 100% R3	5.53 (1.31 - 9.75)	19.41 (14.25 - 24.56)	-
	E 50% R1	-	-	6.068
	E CTL R2	-	-	-3.56

No differences between the samples of the non-diluted effluent (E -100%) collected from the first to the third passage were recorded. Nevertheless, the toxicity of the treated effluent never attained the level of the non-treated sample, being about 10 times lower. The 50% dilution, as well as the passage on the CW contributed for a great reduction of the toxicity, from the first to the third passage. The low residence time may explain why the CW was not more efficient in reducing the toxicity of the OOMW.

3.3 *Raphidocelis subcapitata* growth inhibition test

Despite some microalgae, such as *Scenedesmus sp.,* have been used for treating OOMW (Di Caprio, Altimari and Pagnanelli, 2015), in this study *R. subcapitata* showed to be highly sensitive to OOWM. Andreozzi et al, (2008) found that dilutions of OOMW lower than 1:160 were totally inhibitory to *R. subcapitata* growth. Fiorentino et al, (2003) found two phenolic compounds of the OOMW able to cause high toxicity to *R. subcapitata.* Babíc et al, (2019) also found *Chlorella vulgaris* to be sensitive to OOWM and obtained an EC_{50} of 5.20% for the exposure to raw OOMW.

By looking to the EC_{50} values the results of this study showed a big reduction of toxicity, for the non-diluted effluent, compared to the raw OOWM. Removal of the phenolics compounds during treatment, both by adsorption to the substrate and by microbial degradation, it was likely the cause for the reduction in toxicity since it is known that some of this substances cause inhibition to *R. subcapitata* (Fiorentino *et al.*, 2003).The 50% dilution of the OOMW and its passage through the CW has almost completely abated its toxicity.

Table 4: EC_{10}, EC_{20} and EC_{50} values recorded for growth inhibition of *Raphidocelis subcapitata* exposed to raw OOMW and to OOMW treated by the CW on experiments 2 and 3.

		EC10	EC20	EC50
	OOMW	38.2 (24.2 - 52.3)	46.7 (34.2 - 59.2)	65.6 (55.5 - 75.8)
3	E 100%	44.3 (36.8 - 51.7)	-	-
	E 50%	73.6 (69.0 - 78.1)	80.9 (77.4 - 84.4)	95.3 (93.8 - 96.8)
	E CTL	-	-	-
4	E 100%	35.2 (13.7 - 56.7)	51.7 (31.4 - 71.9)	99.3 (74,4 - 124,1)
	E 50%	71.9 (63.0 - 80.7)	108.7 (98.9 - 118.4)	-
	E CTL	-	-	-

3.4 Immobilization assay with *Daphnia magna*

The acute immobilization assay with *D. magna showed no immobilization* for all the 3.5 samples, and corresponding dilutions.

Different studies showed that the exposure of *D. magna* to OOMW causes mortality to these organisms. Paixão et al, (1999) evaluated the toxicity of various OOMW with a battery of assays. This study reported EC_{50} ranging from 1.08 to 6.83% for *D. magna* after an exposure of 24h. Babić et al, (2019) also reported OOMW to be highly toxic with EC_{50} of 1.43% at 48h of exposure. Fiorentino et al, (2003) tested the different phenols that constitute OOMW and they found two of the most common phenolic compounds; catechol and hydroxytyrosol, to be toxic for *D. magna*. Aggelis et al, (2003) found an LC_{50} of 2.5%, after 48h of exposure, which remained constant even after a treatment with fungi. The persistence of the toxicity was explained by these authors as being possibly caused by the substances resulting from phenols degradation. The lack of an acute effect on *D. magna* in this study was consistent with the results of the toxicity tests with *A. fischeri* and *R.subcapitata* and demonstrated the ability of the CW to reduce the toxicity of the OOMW.

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3.5 Overall treatment effectiveness discussion

As previously mentioned, vertical SSFCW have already been used for the treatment of OOMW. Achak et al, (2019) tested a CW composed of gravel and soil, with a mix of three plant species, *Phragmites australis*, *Typha latifolia* and *Arundo donax*. The CW was built in the field, in a semi-arid region, where temperatures varied between 5º to 45º C. The OOMW was diluted and previously pre-treated with a sand filter to remove part of the organic pollutants. The retention time was two days. The author's recorded reductions of 62.5% for total nitrogen (TN), 90.4% for ammonium, 97.5% for total phosphorous (TP) and 99.1% for total chemical oxygen demand (COD), with the combination of the sand filter and the CW treatment. Herouvim et al, (2011) built, on the field, a series of four CWs with a substrate composed of cobbles, gravel and sand, planted with *Phragmites australis*. The OOMW was pre-treated with a trickling filter, which removed part of the organic content, phenols and in smaller scale nutrients. These CWs had a retention time of five days. They obtained removals of 74% of COD, 77% of phenols, 76% of TN, 74% of ammonium and 87% of phosphates. Yalcuk et al, (2010) used a CW built with sand, zeolite and gravel. The CW was planted with either *Typha latifolia* or *Cyperus alternatifolius* and had a retention time of three days. Removals of 73.46% and 73.91% for COD, 49.06 and 37.38 for NH4–N and 95.43% and 95.93% for PO4–P, respectively were achieved for the CW planted with the species above mentioned, respectively. Dordio et al, (2013) used

Phragmites australis in a CW with a substrate composed of gravel and LECA® and retention times of 6 days. Diluted OOMW was treated and removal percentages of 80.3 ± 5.2 % of phenols and 92.5 ± 4.1 % of COD were obtained for a retention time of 6 days. The authors of this study confirmed that the CW treatment was not enough to comply with EU rules, for discharges of wastewaters. In the current study *Typha latifolia* was able to support even the raw OOMW. These was expected as these plants were chosen due to their ability to withstand similar conditions in their habitat, being also a plant that was already used in other studies (Yalcuk, Pakdil and Turan, 2010; Achak *et al.*, 2019). However, the ability of the CW facilities to decrease the toxicity of the OOMW was few addressed in these studies. On this work, it was shown a reduction of the toxicity of the treated OOMW compared to the raw OOMW. Enhanced retention times, must be tested as they can have a greater contribution to deal with the OOMW, improving its quality. The reduction of toxicity and the correction of the pH could contribute for a safe use of this treated wastewater for irrigation purposes.

4 Conclusion

The vertical SSFCW was able to neutralize the pH and reduce most of the acute toxicity of the OOMW to *Daphnia magna*, despite the increase in conductivity, and reduce sub-lethal toxicity to *R. subcapitata*. A reduction on the toxicity to *A. fischeri* was also recorded, although without improvements related with the maturity of the system.

The usage of other types of soil should be tested to understand if it is possible to reduce the conductivity, as we consider that the artificial OECD soil and its components had a contribution for the increase in this parameter.

Chapter III – The potential use of the wetland substrate as soil fertilizer

1 Introduction

One of the approaches proposed to manage OOOW is through their valorisation for fertirrigation of agricultural soils (Basheer *et al.*, 2019). The irrigation of olive groves, for example, with OOMW has been proposed to reuse the high content of nutrients of these wastewaters, as well as of water, in areas suffering with the scarcity of freshwater for irrigation, as is the case of several countries in the Mediterranean region (Bedbabis *et al.*, 2015).

Many studies have been conducted showing that irrigation with OOMW can alter soil properties depending on its characteristics. The increase of soil conductivity, metal concentrations, salinity and the decrease of pH have been reported as some of the long-term negative effects (Bedbabis *et al.*, 2015; Ayoub *et al.*, 2016).

The impacts of the irrigation with OOMW on the soil biota is also an important subject. The application of OOWM on soil seems to stimulate bacterial activity and soil respiration, thanks to the addition of organic matter. The toxic effects recorded seemed to depend on soil characteristics and concentrations used. In soils that are able to adsorb the phenolic compounds, the bacterial communities are only slightly affected by the toxicity of this wastewater (Kotsou *et al.*, 2004; Mekki, Dhouib and Sayadi, 2006; Karpouzas *et al.*, 2010; Justino *et al.*, 2012). Also, the addition of ammonium and the change in soil pH leads to changes in ammonia-oxidizing bacteria proportions, favouring some taxa in particular (Kotsou *et al.*, 2004; Mekki, Dhouib and Sayadi, 2006; Karpouzas *et al.*, 2010; Justino *et al.*, 2012). The effect of OOMW on soil invertebrates has been investigated in a few studies. Kurtz et al, (2015) found that OOMW can have a duality of positive and negative effects on the microarthropods community. While the toxicity seems to cause inhibition of the most sensible species such as those from the *Oribatida* genus, the increase of organic matter on the soil leads to a population increase of some species of *collembolans*. Hentati et al, (2014) assessed the avoidance behaviour of earthworms and springtails exposed to soil irrigated with OOMW. Earthworms tended to avoid the soil irrigated with OOMW. *Collembolans* presented the same behaviour but they also found springtails to be less sensitive. Overall the use of OOMW for irrigation seemed to cause alterations on soil communities by supressing sensitive organisms and given better growth conditions to those able to withstand the short-term toxicity.

The usage of OOMW to irrigate olive trees has been studied showing little effects on the quality of the olives harvested (Magdich *et al.*, 2015; Basheer *et al.*, 2019) - However, the tendency to increase soil salinity may pose a threat to the quality of the olive oil produced, due to reduced water and oil content on olives (Tietel *et al.*, 2019). Furthermore, it has been shown that the phenolic compounds responsible for OOMWs phytotoxicity can lead to inhibitory effects on seeds germination and reduce the growth of a variety of crops (Muscolo *et al.*, 2010; Asfi, Ouzounidou and Moustakas, 2012; Fendri *et al.*, 2013; Massoudinejad, Arman and Aghayani, 2014; Rusan *et al.*, 2015). Nevertheless, it has been suggested that these molecules are rapidly degraded, when in contact with soil and when the optimal conditions for the soil microflora are not compromised , they do not accumulate on soil after successive irrigations with OOMW (Sierra *et al.*, 2001; Chartzoulakis *et al.*, 2010).

On chapter two, a vertical sub-surface flow constructed wetland (VSSCW) was proposed as a more ecological and sustainable approach to treat OOMW. However, problems may appear with the clogging of the substrate which may reduce the flow of water and air and subsequently contribute for the reduced efficiency on phosphate removal through time. These problems may lead to the need of replacement of substrate components when the life cycle of the VSSCW ends (Knowles *et al.*, 2011; Vohla *et al.*, 2011; Ayaz *et al.*, 2012; Fu *et al.*, 2013; Hua *et al.*, 2013). The application of a VSSCW to the treatment of OOMW in rural areas, is facilitated by the seasonal production of olive oil, and subsequently of OOMW. This allows the renewal of the VSSCW substrate at the end of each season. Thus, the usage of the VSSCW substrates as an organic fertilizer for agricultural purposes is one possible management approach since the substrate tends to accumulate nutrients and organic matter, as well as a microbial community that is able to deal with the most toxic compounds of OOMW, such as the phenolic compounds. Further, the application of the CW substrate prevents a direct application of OOMW without a previous treatment and or dilution, and it also may contribute for improving other physical properties of soil, such as its structure (Sierra *et al.*, 2001; Mustafa and Scholz, 2011; Fu *et al.*, 2013).

In this work it was hypothesized that the substrate of VSSCW used to treat OOMW, is not toxic to soil biota and can be used as a soil fertilizer, contributing for the growth of crop species.

2 Material e methods

2.1 Soil substrate

An artificial soil was prepared to be used as a substrate of the ecotoxicological assays performed. This soil is composed by 70% of sand, 20% of kaolin clay and 10% *of Sphagnum* peat (OECD, 1984), and it was used as control. The soil pH was measured following the standard protocol (OECD, 2013). For this purpose 10g of soil were suspended in 50ml of a KCl solution (1M), and after 15 minutes of stirring and 30 minutes of resting, the pH was measured with a pH meter (Hanna Edge Hi 2020).The pH was then adjusted for values between 5.5 and 6.5 by adding 2.5g of calcium carbonate per 10kg of soil. After 24 hours the pH was measured again to confirm the readjustment made to the soil. The maximum water holding capacity (WHC) was determined following the standard protocol (ISO, 2019). For this purpose, plastic cups with the bottom replaced by filter paper were filled with soil and placed in a trail with water for 3 hours. Afterwards the cups were removed from water and placed 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^{o}C for twelve hours and then the dry weight was recorded and the maximum water holding capacity calculated. Both parameters were determined in triplicate.

Before the ecotoxicological assays deionized water was added to adjust soil WHC to 45% of its maximum value.

2.2 *Eisenia fetida* culture conditions

The earthworm *Eisenia fetida* was used as a test organism. They were obtained from laboratorial cultures kept under environmental controlled conditions at the LABRISK in the Faculty of Sciences of the University of Porto. Earthworm are cultured in plastic boxes with a medium composed of peat, horse manure and deionized water and fed with defaunated horse manure (that undergoes two freeze–thawing cycles of 48h, at −20°C and at 65°C, respectively) hydrated with deionized water and oat. Before usage in the assays earthworms were acclimated in OECD soil for 24h.

2.3 Experimental design

After the treatment of OOMW (for more details see Chapter 2), the CW was dismantled. The components of the substrate of the replicates of each CW treatment, were mixed and smashed to reduce the granulometry of the expanded clay pebbles (LECA®). At the end

three substrate mixtures remained: E CTL – resulting from the 3 vertical SSFCW replicates that received only tap water; E 100% - the substrate of the three replicates of the vertical SSFCW that received the non-diluted OOMW and, the E 50% - the substrate of the three replicates of the VSSCW that received the 50% diluted OOMW. Each one of these substrates were integrated in the composition of the OECD soil, replacing the *Sphagnum* peat, thus in a 10% proportion. Both pH and WHC of soils mixed with VSSCW substrates were measure again as explained in 3.2.1 chapter. The pH of all soils was found to be between 5.92 to 6.34.

2.4 Avoidance assay with *E. fetida*

This assay followed the protocol 17512-1 from ISO (ISO, 2008). Four types of soil were used in this assay (for more details please see sections 2.1 and 2.3). Figure 1 describes the dual treatments tested.

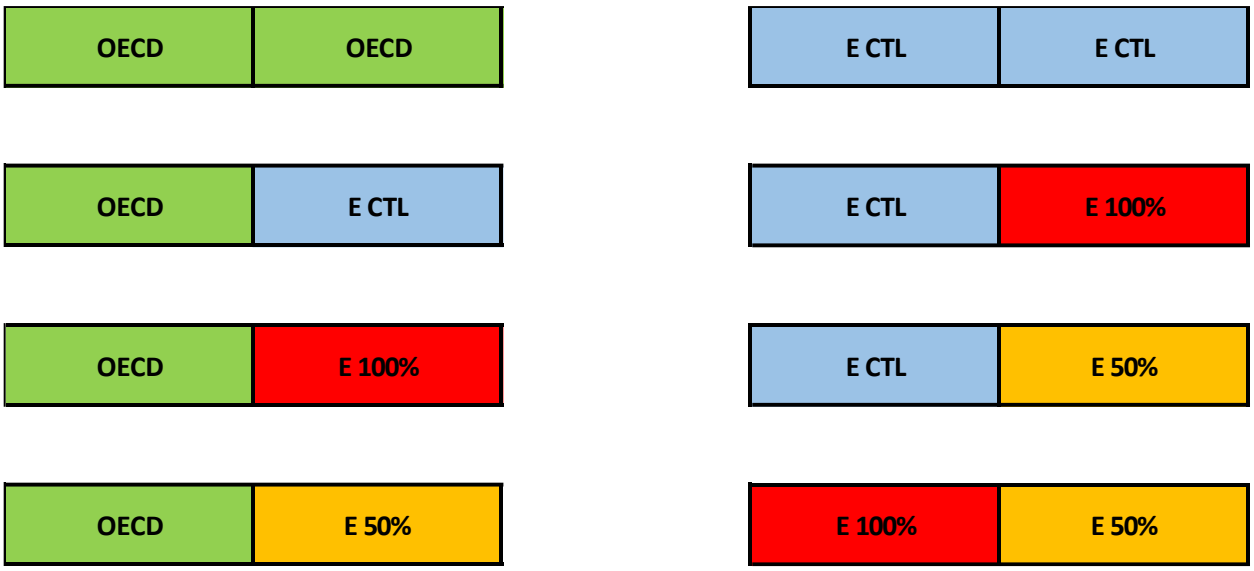

Figure 1 – Schematic representation of the dual combinations of soils tested.

The plastic boxes prepared for each treatment (25.5cm length, 17.4cm width, 6.5 weight) were divided in two equal parts with a card and in each side of each box were placed 250g of the corresponding soil. Ten earthworms weighing between 0.3 and 0.6g were placed in the middle line of each box. When all the boxes were prepared, the cards were removed and the soil gently joined in the middle line. All the replicates were placed in a room with controlled conditions of temperature (21±1ºC) and photoperiod (16 h^{L}:8 h^{D}).

After 48 hours of exposure, the card was placed again in the line dividing both parts of each box and the number of earthworms in each side was counted. A two-tailed Fischer exact test was performed to test the hypothesis of no significant differences between the number of organisms on both sides of the boxes in the OECD/OECD treatment, as well as in the other dual treatments tested.

$$E\% = \frac{Sref - Stest}{N} \times 100$$

The avoidance percentage of each replicate was calculated using the formula above. Sref is the number of earthworms in the reference soil (OECD or ECTL). Stest is the number of earthworms in test soil. N is the total number of organisms in the replicates.

2.5 Seedling growth test with *Lactuca sativa* and *Avena sativa*

This assay was performed according to, an adaption of the protocol OECD nº 208 (OECD, 2006) for seedling growth inhibition test, to assess phytotoxicity and the fertilization potential of the VSSCW substrates on crop species.

Six different treatments with five replicates each were tested (Figure 2).

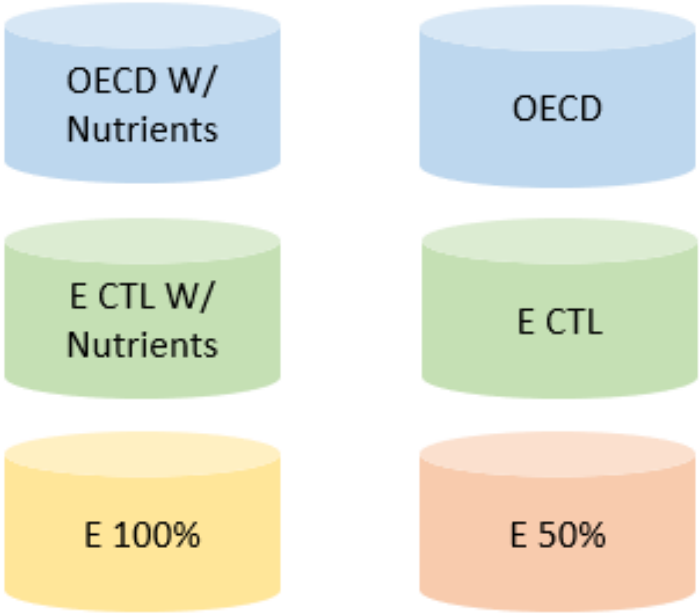

Figure 2: Experimental treatments of the of seedling growth test, each one tested for five replicates.

The test pots had a hole in the bottom, which allowed a cotton rope to pass through, being immersed in a cup filled with deionized water, placed under the test pot. In each test pot (11.7 cm diameter, 6.2 cm height), 200 g of the respective soil were placed (for more details please see sections (2.1 and 2.2). Afterwards, and only once at the beginning of the test, the replicates of one of the OECD and of ECTL treatments received a nutrients solution for marshland plants that was made following the table 1 found in the annexes , in the cup placed in the bottom, whereas the other treatments received deionized water.

Twenty seeds of each test species (*L. sativa* and *A. sativa*, tested individually), obtained from a local supplier and previously inspected for their good condition were added to each test pot and gently buried in the soil surface.

The pots were placed in a growth chamber with photoperiod of 16 h^L:8 h^D and constant temperature of 21±1ºC. Pots were checked each day, to count germinated seeds, and only the first 5 seeds germinated in each pot were left to growth. The others were removed. After 50% of the seeds had germinated on the OECD soil with nutrients the test started and was prolonged for 14 days. The plants were watered every two to three days with dezionized water by adding water on the pots supporting the test pots. At the end of the assays all seedlings in each replicate were cut and the total fresh and dry weights of the biomass above soil was determined. The fresh biomass of each replicate was weighted immediately and then the vegetal material was dried at 60Cº for 24 hours and weighed. Average values and corresponding standard deviation of both parameters (fresh and dry biomass) are presented. Significant differences between treatments were tested with One-Way ANOVAS followed by a Tukey HDS multicomparison test. A level of significance of $p<0.05$ was used. Levene's test was performed previously to check the homogeneity of variances between groups.

3 Results and discussion

3.1 Avoidance assay with *E. fetida*

The validity criteria of the avoidance test with earthworms were accomplished, as there was no mortality in the replicates. Further, no significant avoidance or preference was recorded in the dual test with OECD soil. Despite the great variance between replicates in the same treatment, only in the treatment OECD vs E 100% a significant preference by the E 100% soil was recorded (Fischer Exact Test: $p=0.0124$). In all other combinations of soils, no significant avoidance or preference was recorded for any soil of the dual combinations. These results can be seen in the figure 3.

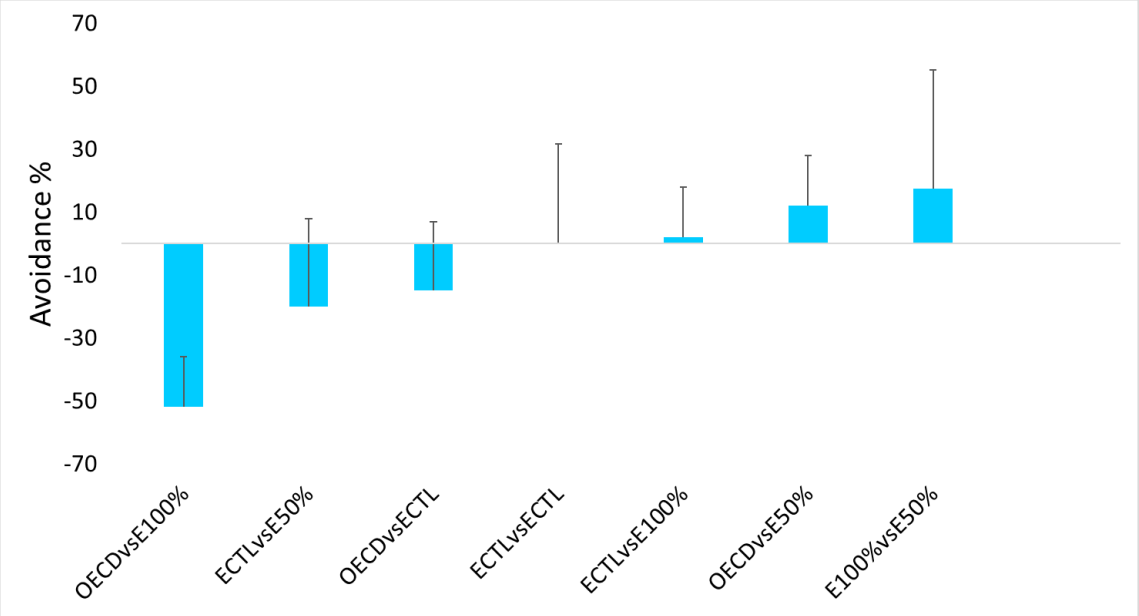

Figure 3: Average avoidance percentage and corresponding standard deviation for the OECD soil mixed with sphagnum peat or with the different substrate mixtures from the VSSCW pilot used for OOMW treatment experiences.

The lack of a significant avoidance between the OECD soil vs the E CTL soil (soil with CW substrate that received distilled water) showed that the CW substrate *per se* did not compromise the habitat function of the soil for earthworms. The preference for E 100% over OECD might also showed that the polyphenols enriched substrate was not toxic to earthworms and contributed for an enrichment of soil with organic matter that was favoured by these organisms. In fact, OOMW applied to a test soil in concentrations of 50g per 100g of test soil have been found to cause mortality of 80% to *Eisena fetida* after an exposure of 72h. While lower concentrations of 12g per 100g of soil caused neurotoxic effects, induction of oxidative stress and genotoxic effects (Campani *et al.*, 2017). Avoidance assays with earthworms also found them to avoid soils that have been irrigated for a long-term with OOMW (Hentati *et al.*, 2014).

OOMW contains groups of bacteria and fungi adapted to the conditions of OOMW and are capable of degrading phenolic compounds (Ntougias, Bourtzis and Tsiamis, 2013). Species of this communities have been isolated and used to treat OOMW in several studies (Di Gioia *et al.*, 2002; Ben Othman *et al.*, 2008; Badr *et al.*, 2016). The reduction in toxicity may have been caused by the activity of indigenous microbiota of the OOMW. However, the amount of polyphenol and other OOMW components added with the substrate is always lower than the amount that attains the soil through direct irrigation, and

this may have contributed for not compromising the habitat function of the soil to earthworms.

E. fetida are known to prefer soils with higher quantities of organic matter, which they use to feed on (Garg, Gupta and Satya, 2006; Askham, 2007). The accumulation of organic matter in the E 100% substrate could be a reason for the earthworm preference for this soil over OECD soil, as seen in studies with this specie. (Fu *et al.*, 2013)

3.2 Seedling growth test with *Lactuca sativa* and *Avena sativa*

Figures 4, 5, 6 and 7 show the results of the growth in both plants. One Way ANOVA showed significant differences in all treatments ($P<0.05$), both for *Lactuca sativa* fresh weight ($F= 36.90$; d.f=28, 23; $p<0.05$) and dry weight ($F= 8,09$; d.f=28, 23; $p<0.05$) and also for *Avena sativa* assay in fresh weight ($F= 26.68$; d.f= 25, 20; $p<0.05$) fresh and dry weight ($F= 23.56$; d.f= 25, 20; $p<0.05$).

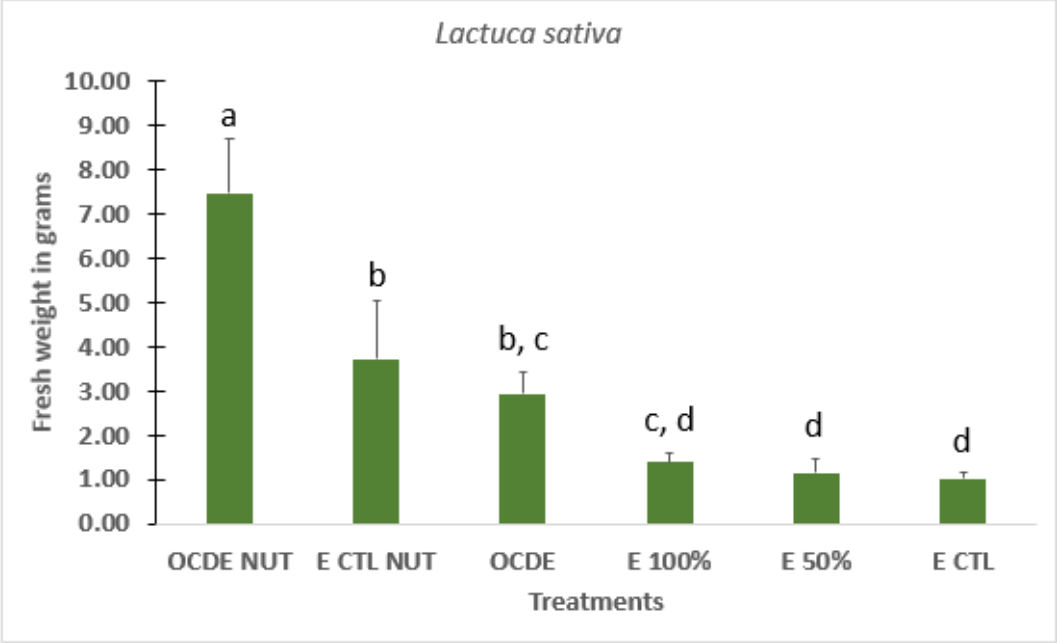

Figure 4: Average fresh weight of *Lactuca sativa* above the soil surface for each treatment tested. Different letters denote significant differences between treatments ($p<0.05$), according to the Tukey HSD multiple comparison test. Error bars represent standard deviation between replicates.

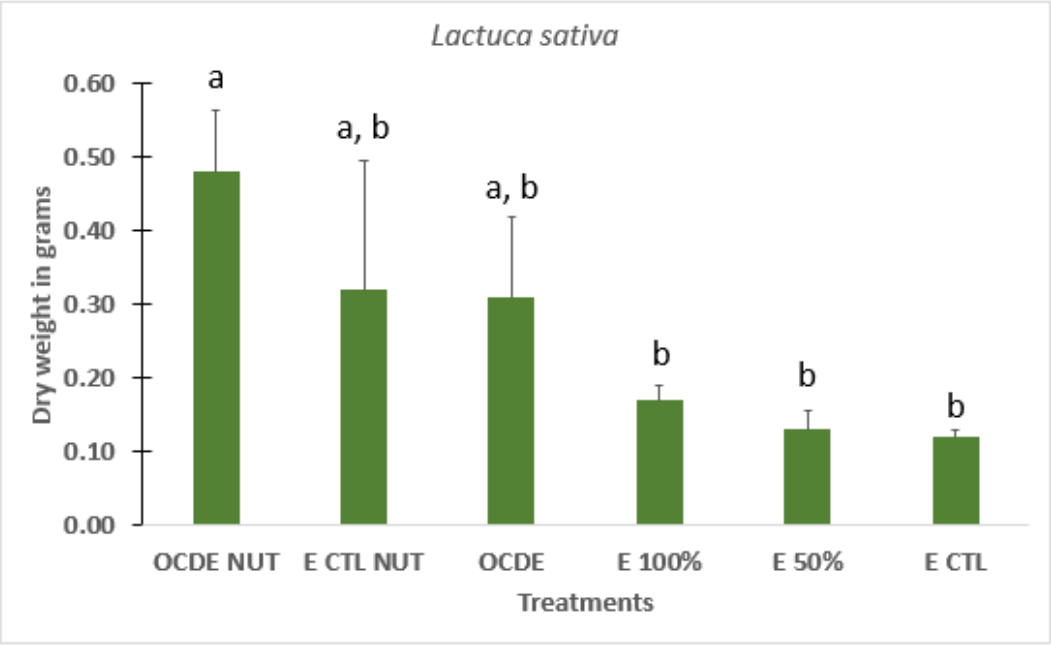

Figure 5: Average dry weight of *Lactuca sativa* above the soil surface for each treatment tested. Different letters denote significant differences between treatments ($p<0.05$), according to the Tukey HSD multiple comparison test. Error bars represent standard deviation between replicates.

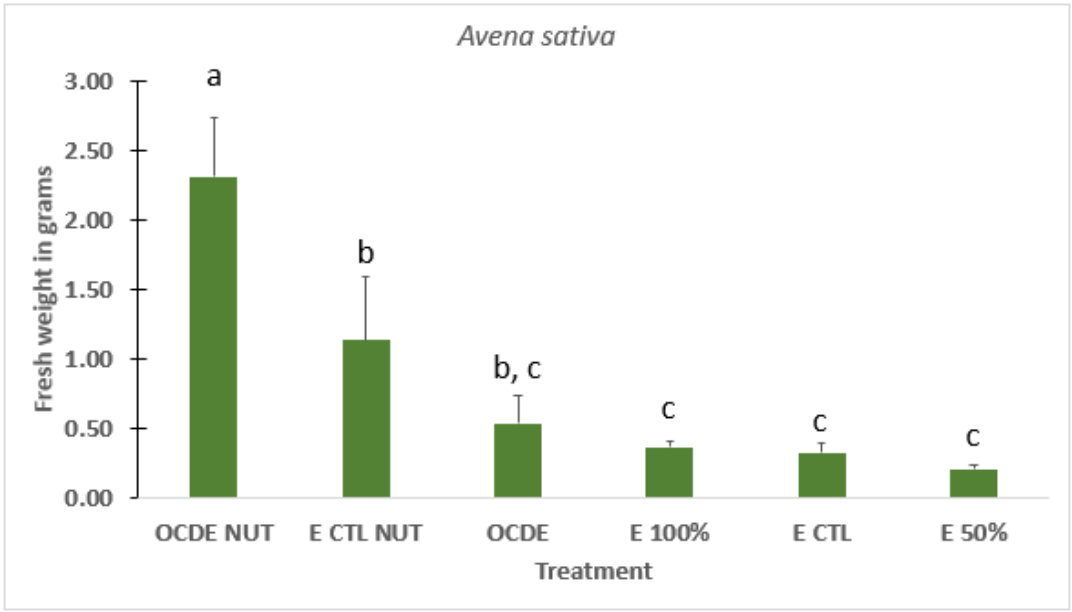

Figure 6: Average fresh weight of *Avena sativa* above the soil surface for each treatment tested. Different letters denote significant differences between treatments ($p<0.05$), according to the Tukey HSD multiple comparison test. Error bars represent standard deviation between replicates.

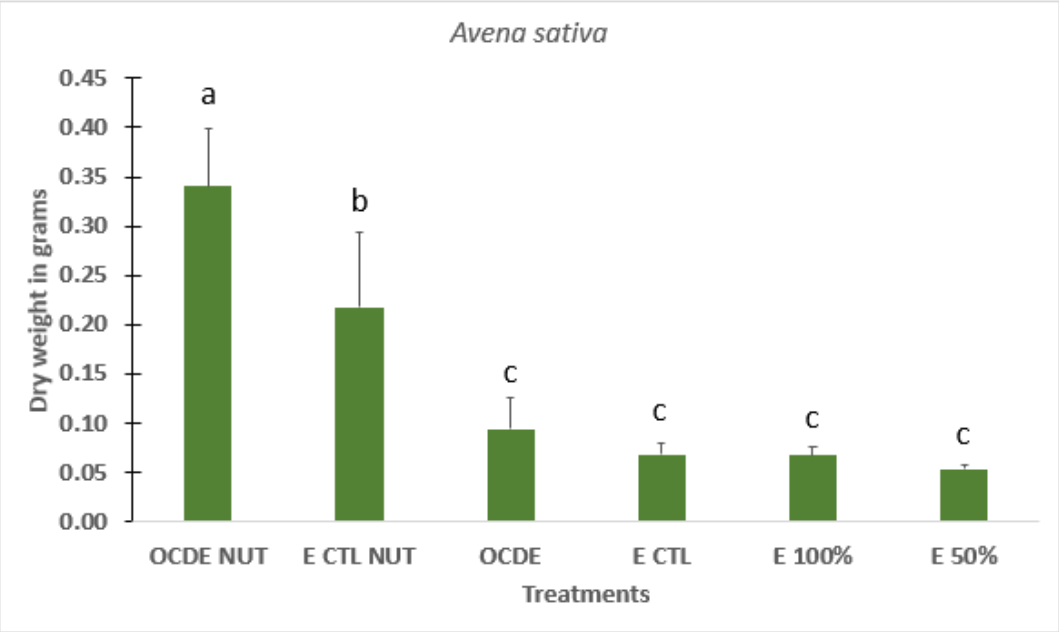

Figure 7: Average dry weight of *Avena sativa* above the soil surface for each treatment tested. Different letters denote significant differences between treatments ($p<0.05$), according to the Tukey HSD multiple comparison test. Error bars represent standard deviation between replicates.

In both assays, no significant differences were recorded between the dry weight of plants recorded in the treatments with VSSCW substrates mixed with OECD soil and the E CTL without nutrients (Tukey: $p<0.05$). This demonstrated once again that the substrate per se and the substrate with OOMW components did not inhibit the growth of plants, i.e. the substrate of the different VSSCW treatments was not phytotoxic. Since previous studies have shown that phenolic compounds cause inhibition on plant growth (Muscolo *et al.*, 2010; Asfi, Ouzounidou and Moustakas, 2012; Massoudinejad, Arman and Aghayani, 2014) we can concluded that the substrate of the VSSCW did not contribute with an amount of these compounds able to promote phytotoxic effects on the plants tested.

Even though the growth does not seemed to be inhibited by phenolic compounds the soils fertilized with VSSCW substrates were, overall, less productive than the soils that received inorganic nutrients. This could be reverted by a lag time between the soils preparation and the addition of seeds to soils (i.e. the beginning of the test), as this lag time is likely needed to let the microbial community to start the mineralization of organic matter added to soils through the VSSCW substrates. This is particularly important if we take into account that the soil microbial community is generally poor in OECD soil, giving that only the sphagnum peat, or in this case the substrates, were the only source of microorganisms added to this soil. This makes of utmost importance the testing of these

substrates with a natural soil. Another aspect is the changes in soil characteristics induced by the fertilization with the CW substrates. Overall the organic matter contained in the CW soils should increase the soil fertility, other changes such as in the soil structure, water retention and microbial community need to be better studied, but they can also be positive in more degraded agricultural soils.

4 Conclusion

Both the growth inhibition assay with *L. sativa* and *A. sativa* and the avoidance assay with *E. fetida* showed that the substrates of the VSSCW both loaded and non-loaded with OOMW presented no phytotoxicity/toxicity and can be safely disposed on soil, being a good alternative to the direct irrigation of soils with OOMW. However more experiments and new experimental approaches are needed to confirm the fertilization potential of these substrates.

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Annexes

Table 1A – Salts and their masses needed for 1 L of stock nutrient solution for marshland plants.

	Salt	Masses for 1 L		
1	KNO_3	15.17	g	Potassium nitrate
2	$Ca(NO_3)_2$	16.41	g	Calcium nitrate
or	$Ca(NO_3)_2.4H_2O$	23.62	g	Hydrated Calcium Nitrate
3	$NH_4H_2PO_4$	5.75	g	Ammonium Hydrogen Phosphate
or	KH_2PO_4	6.80	g	Potassium hydrogen phosphate
or	$MgSO_4$	3.01	g	Magnesium Sulphate
4	$MgSO_4.7H_2O$	6.16	g	Magnesium Sulfate Hexahydrate
5	KCl	0.37	g	Potassium chloride
6	H_3BO_3	0.15	g	Boric acid
7	$MnSO_4.H_2O$	0.03	g	Hydrated Manganese Sulfate
8	$ZnSO_4.7H_2O$	0.06	g	Zinc Sulphate Hepathydrate
or	$ZnCl_2$	0.03	g	Zinc Chloride
9	$CuSO_4.5H_2O$	0.01	g	Copper Sulphate Pentahydrate
10	$(NH_4)_6Mo_7O_{24}$	0.06	g	Ammonium molybdate
or	$(NH_4)_6Mo_7O_{24}.4H_2O$	0.06	g	Ammonium Molybdate Tetrahydrate
11	$Fe(NO_3)_3.9H_2O$	0.81	g	Hydrated Iron Nitrate
or	$FeCl_3.6H_2O$	0.54	g	Iron chloride
12	$Na_2H_2EDTA.2H_2O$	0.74	g	EDTA

(a) - Weigh the indicated masses
Add the indicated masses to a 1L gobelle
Add 800 ml of dionized water
Dissolve the salts using na agitacion plate
Add water until 1L