Universidade de Aveiro Departamento de Biologia
Ano 2018

Ana Filipa Silva Domingues

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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Mestre em Toxicologia e Ecotoxicologia, realizada sob a orientação científica da Doutora Joana Luísa Lourenço Estevinho Pereira, Bolseira de Pós-Doutoramento do Departamento de Biologia da Universidade de Aveiro, e coorientação da Professora Doutora Ruth Maria de Oliveira Pereira, Professora Auxiliar com Agregação da Faculdade de Ciências da Universidade do Porto.

Aos meus avós…

o júri

presidente

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palavras-chave

Corbicula fluminea, efluentes de azeite, biorremediação, biofiltração, bioadsorção, compostos fenólicos

resumo

O azeite é considerado um dos principais produtos alimentares típicos da gastronomia mediterrânea, pelo que a sua produção alimentar é particularmente elevada nesta região. Apesar da sua importância económica e gastronómica, a indústria do azeite suscita algumas preocupações ambientais no que diz respeito aos efluentes, vulgarmente conhecidos por águas ruças, resultantes do processo de extração. As águas ruças são constituídas por misturas complexas de compostos orgânicos, entre os quais se destacam os polifenóis. A reconhecida toxicidade destes compostos, para além dos elevados volumes de efluentes gerados em curtos períodos de tempo devido à sazonalidade da produção de azeite e, por último, à inexistência de um tratamento efetivo para estes efluentes, torna as águas ruças num problema ambiental atual. Ao longo dos anos, vários tratamentos químicos e biológicos têm sido desenvolvidos para reduzir tanto a toxicidade como a cor destes efluentes, no entanto ainda não foi encontrada uma solução que satisfaça completamente estes critérios. Neste contexto, é relevante testar novas abordagens de tratamento, tendo sido identificada a possibilidade de usar a capacidade de filtração e acumulação de alguns bivalves para o efeito. Assim, o objetivo central da presente dissertação foi explorar a viabilidade de *Corbicula fluminea* como agente remediador de efluentes de lagar de azeite através da caracterização do seu potencial enquanto biofiltrador e do potencial adsorvente das suas conchas. A amêijoa asiática *C. fluminea* é um reconhecido bivalve invasor de sistemas de água doce, estando disperso um pouco por todo o mundo. Apesar da sua elevada dispersão e consequentes impactos ambientais negativos, a sua elevada capacidade filtradora e a sua tolerância e capacidade bioacumuladora de alguns contaminantes torna-a numa opção interessante enquanto potencial agente biorremediador. Os resultados mostram que a amêijoa asiática, quando utilizada viva, foi efetivamente capaz de remover uma fração significativa de contaminantes orgânicos deste efluente, levando a uma diminuição significativa da sua toxicidade ambiental, conforme resultados de bioensaios efetuados com espécies indicadoras. A esta remoção corresponderam inversamente alterações na composição molecular do corpo mole e das conchas, sugerindo os resultados ainda que poderá haver sedimentação de compostos promovida pela produção de pseudofezes. Quando aplicadas isoladamente, as conchas parecem não ter capacidade significativa de remoção de compostos do efluente. O potencial biorremediador deste bivalve seria adicionalmente valorizável já que o seu aproveitamento a nível industrial colmata, em certa medida, os efeitos nefastos e prejuízos económicos associados à sua natureza invasora em ecossistemas de água doce, e infestante em indústrias dependentes deste recurso.

keywords

Corbicula fluminea, olive oil mill wastewaters, biorremediation, biofiltration, biosorption, phenolic compounds

abstract

Olive oil is considered one of the main food ingredients in the Mediterranean countries, and its worldwide production is predominant in this region. Despite its paramount economic and gastronomic importance, the olive oil industry poses some environmental concerns due to olive oil mill wastewaters (OOMW) produced as a result of the extraction process. OOMW are composed by complex mixtures of organic compounds, including polyphenols. The recognized toxicity of these compounds, the high volumes of wastewaters generated in short periods of time due to the seasonality of the process, and, at last, the inexistence of an effective treatment to these wastewaters renders OOMW an actual and current environmental issue. Through the years, several chemical and biological treatments have been developed to reduce both the colour and toxicity of these effluents, however, a solution that completely meets these criteria has not been found yet. Therefore, it is relevant to test new treatment approaches, and the opportunity of using the filtration and accumulation capabilities of some bivalves has been raised as the rational for the present studies. Thus, the main objective of the present dissertation was to explore the viability of the Asian clam, *Corbicula fluminea* as a remediation agent of OOMW through the characterisation of its potential as a filter-feeder, and the potential of its shells as a biosorbent biomaterial. *C. fluminea* is a recognized freshwater invasive bivalve worldwide outside its native range in Eastern Asia. Despite its high dispersion and consequent negative environmental impacts, its capacity of filtering, tolerate and bioaccumulate some contaminants may render the Asian clam an interesting option as a bioremediation agent. Results show that living *C. fluminea* were able of significantly removing a fraction of organic contaminants of this wastewater, significantly reducing its environmental toxicity according to the results of bioassays performed with indicator species. This removal inversely corresponded to changes in the molecular composition of the soft body and the shells, the results suggesting further that a relevant part of the removed compounds may have been deposited through the production of pseudofaeces. When applied *per se*, the clam shells do not appear to have significant capacity to remove compounds from OOMW. The bioremediation potential of this bivalve would be further valued as its industrial use compensates, to a certain extent, the deleterious effects and economic losses associated with both its invasive nature in freshwater ecosystems and its biofouling activity in water-dependent industries.

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CHAPTER 1. Introduction

1.1. Olive oil mill wastewaters and related environmental problems

The Mediterranean region is known worldwide for its production of olive oil. The utmost importance of this industry is noticeable in the economy of countries like Portugal, Spain, Italy and Greece, as they are considered the four major European producers of olive oil (IOOC 2017). Notwithstanding, this industry poses some environmental problems that are worth mentioning, such as a huge water footprint and the production of high volumes of extremely toxic wastewaters – olive oil mill wastewaters (OOMW) (Gebreyohannes et al. 2016), that are released to water and soil, affecting the biota and ultimately severely impairing ecosystems (Priac et al. 2017; Tsioulpas et al. 2002; Mekki et al. 2008; Azaizeh et al. 2011; Dhouib et al. 2006). The problem is intensified with the lack of legislation and surveillance associated with an absence of proper treatment strategies to deal with OOMW: while chemical treatments efficiently remove the colour of OOMW, biological treatments are more suitable to remove the toxicity of these effluents. In general, biotreatments are still underexplored in the context of industrial wastewaters, despite their sustainable, environmentally friendly and cost-efficient nature compared to physicochemical treatments (Paraskeva & Diamadopoulos 2006). In the case of OOMW, fungi are considered the most powerful bioremediation agents (Duarte et al. 2014). Chapter 2 of the present dissertation constitutes a wide revision on the current valuation and treatment strategies suitable to address OOMW, also focusing its production processes, chemical characterisation and hazardous potential. Therefore, please refer to Chapter 2 for further considerations on these topics and for a complete contextualisation of the developed experimental studies.

Provided the contextualisation of the environmentally hazardous matrix and its treatment approaches, it is consistent to now provide the overall question addressed in the present studies: can the invasive bivalve *Corbicula fluminea* be used as a bioremediation agent of olive oil mill wastewaters? The acknowledgement of the powerful filtration process inherent to this bivalve comparing to other bivalve species is already described in the literature (Castro et al. 2018), and the shell compartment has also been demonstrated to be a suitable sorption matrix for metallic contaminants (Gifford et al. 2004). Thus, the possibility of developing filtration- and sorption-based treatments for OOMW was focused here. For a proper support to the research question, the following section presents a summary on the ecophysiological traits, nuisance potential and management (mostly control) strategies for this clam.

1.2. The invasive bivalve *Corbicula fluminea*

Corbicula fluminea, commonly designed by Asian clam, is an invasive species belonging to the Bivalvia class of the Mollusca phylum (DAISIE 2009a). This species shows high variation in the phenotype of the shell at the level of morphology, colour and ornamentation, which may have constrained its correct taxonomic classification in previous studies (Rosa, Pereira, et al. 2014).

The morphology of *C. fluminea* comprises typical features, most of them easily identified in Figure 1.1. The shell is particularly differentiable as it has a rough structure ornamented with concentric and elevated ribs (8 to 11 ribs.cm^{-1}) (Ilarri et al. 2015; Reyna et al. 2013). Shells are of a brownish ochre on the external side and ivory on the internal side; the two strongly pigmented syphons are also typical (Reyna et al. 2013).

Figure 1.1. The bivalve *Corbicula fluminea*, commonly known as Asian clam (Ana Domingues®).

The Asian clam is a hermaphroditic species with the ability of both cross and self-fertilization (Morton 1982). The reproduction process (Figure 1.2) can be described within the following steps: first, fertilization occurs in the paleal cavity, where larvae are incubated for a period; after that, they are released to the water column as pediveligers (small juveniles) of reduced size (approximately 250 µm) and mobility, so they settle near vegetation or sediments. At this stage, juveniles are fully developed with shell, adductor muscles, foot, statocysts, gills and digestive system, presenting a D-configuration in body structure. Until they are considered adults they go through a maturation period that can last for 3 to 6 months, during which their shell increases to lengths between 6 and 10 mm. After this period, the shell of adult clams can reach a length between 15 and 30 mm in the first year. Life span of the clams is between 1 to 4 years, normally with two reproduction cycles per year (spring to summer, and then late summer to autumn) if there aren't environmental interferences like temperature or seston composition (Sousa et al. 2008; Viergutz et al. 2012; Gherardi 2007).

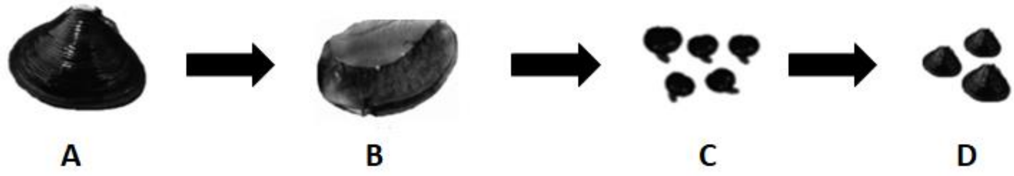

Figure 1.2. Life cycle of *Corbicula fluminea*: A) adult specimen; B) inner demibranch with larvae; C) small juveniles and D) small adults. Adapted from Sousa et al. (Sousa et al. 2008).

Geographically, *C. fluminea* is a globally widespread bivalve through freshwater ecosystems which are in an oligotrophic to eutrophic state, in habitats that are composed by sand, mud or gravel (Araujo et al. 1993; DAISIE 2009b). The dispersal of the Asian clam from its native range in Southeast Asia started in the beginning of the twentieth century when the bivalve spread out to North America (Rosa, Pereira, et al. 2014; Mackie & Claudi 2010). Then, at the beginning of 1970, the clam reached South America, followed by Europe around the 1970s/1980s and finally North Africa at the beginning of the present century (Ferreira-Rodríguez et al. 2016; Mackie & Claudi 2010). The initial introduction of the Asian clam in North America was attributed to Chinese immigrants that used the clam as food. Anthropogenic practises such as global trade, agriculture, aquaculture and recreational transports/activities represent the main passive introduction route by which the clams are dispersed all around the world (Sousa et al. 2008; Gama et al. 2016).

The main dispersal mode of this bivalve is based on the pediveliger and juvenile forms suspended in the water column, as this allows their transportation by water currents singly or attached to different vectors (organic, like algae and macrophytes; and synthetic, like boats and other fishing/navigation objects), and fast colonization of sites downstream mainly in lotic systems. The small size and mass of both pediveligers and juveniles enhances long-distance dispersal without great energetic consumption. In addition, clams flotation is a fundamental phenomenon promoting expansion: mucocytes, a group of modified cells in the demibranchs, are responsible to produce a mucilaginous drogue line that aids the drifting of these organisms when exposed to mechanical forces, like waves and water currents. This mucous drogue line is produced while clams are juveniles and young adults of no more than 14 mm shell length (Rosa et al. 2012; Rosa, Pereira, et al. 2014). Despite these advantages, adult *C. fluminea* are incapable of attaching to physical structures since they neither produce a byssus nor possess alternative adhesive structures (Mackie & Claudi 2010).

The success of the invasion process depends on the biological traits of the Asian clam: fast growth and high fecundity rates; rapid sexual maturation; short life spans and capability of reproduction by hermaphroditism with self-fertilization, which means that only one individual has the ability of starting a new population in a new habitat (Rosa, Pereira, et al. 2014; Ferreira-Rodríguez et al. 2016; Crespo et al. 2017). This bivalve also presents a great genetic and phenotypic variability, broad physiological tolerance towards some abiotic factors and, most importantly, an opportunistic behaviour (Sousa et al. 2008). Regardless of the importance of all these traits, it is consensual that the filtration capability of *C. fluminea* also plays a preponderant role in a successful invasion and its consequent ecosystem impacts (J. Gomes et al. 2018; Sousa et al. 2014; Sousa et al. 2009).

In what concerns the water column, the Asian clam regulates the presence of seston as a non-selective filter-feeder (Marescaux et al. 2016; Vaughn & Hakenkamp 2001). The filtration process generally depends on several parameters such as temperature, water velocity, algae concentration, type of suspended particles available and also bivalves' body size, density, reproduction and growth cycle (Marescaux et al. 2016; Way et al. 1990). It is relevant to note that part of the filtered particles removed by bivalves from the water column are transferred to the sediments through pseudofaeces (Lorenz & Pusch 2013; Marescaux et al. 2016). Pseudofaeces constitute a viable organic food resource to the benthic and pelagic biota (Sousa et al. 2008; Way et al. 1990) and a key player in the sedimentation process in freshwater ecosystems (Vaughn & Hakenkamp 2001). Besides, the Asian clam can switch between filter and pedal-feeding, according to the food concentration available (Marescaux et al. 2016; Vaughn & Hakenkamp 2001). Pedal feeding not only presents an important role for the sediment-water interface where bivalves dwell, but also provides higher energetic resources than filter-feeding (Vaughn & Hakenkamp 2001). From a practical point of view regarding the use of the clam's filtering capacity in bioremediation, pedal-feeding allows the exploitation of deposited residues along with those suspended in the water column which demonstrates a high feeding plasticity (Castro et al. 2018).

1.3. Brief notes on the nuisance potential and control strategies of *Corbicula fluminea*

The establishment of *C. fluminea* in non-native habitats may prompt some negative consequences in the biotic interactions and also in the hydrology and biogeochemistry of the ecosystem, for example: introduction of parasites and diseases; competition of food and habitat resources against native species (unionids); alteration of abiotic conditions;

and biofouling, in both natural channels and industrial systems (Sousa et al. 2008; Ferreira-Rodríguez et al. 2016; Crespo et al. 2017; Rosa et al. 2011). For instance, Ferreira-Rodríguez et al. (2016) conducted a study in which they addressed the influence of *C. fluminea* over the native species *Unio delphinus*, and the conclusions were that an increase in *C. fluminea* density induced a stressful environment for *U. delphinus*, as the Asian clam competed for the available food resources in such a way that the native population was only able to rely on their own energetic reserves. The decrease in the carbohydrates concentrations corroborated the previous statement, as they are known physiological biomarkers that reflect the health condition in freshwater bivalves.

Biofouling is the process of accumulation of a biological population (microorganisms, plants, algae, animals) in a non-natural surface, mostly in industrial equipment's and systems, causing its deterioration and degradation (Mackie & Claudi 2010). Some of the most reported damages happen on pumphouse structures; raw water systems; piping of construction materials; fire protection systems; drainage and sumps; heating, ventilation and air-conditioning systems; and on artificial facilities, namely on dams, reservoirs, aqueducts, drinking water plants and fish diversion structures (Mackie & Claudi 2010). This is a major issue since biofouling by the Asian clam already caused damage in the economy of many industries worldwide (Pimentel et al. 2005; Darrigran 2002). In the United States of America, the economic losses caused by the *C. fluminea* were highly significant. Despite its presence in Portugal, the Asian clam hasn't reached yet all Portuguese watersheds and, when present, has not caused problems with the degree of severity as registered in America (Rosa et al. 2011). A possible explanation for this fact is that this bivalve could still be in its lag time phase, a period after the colonization of a habitat where a population maintains its stability at low abundance records. If this hypothesis is correct, it is only a matter of time until the species starts proliferating out of control. Another hypothesis is that *C. fluminea* is prone to die-offs, especially in the summer, which naturally control populations in newly invaded aquatic ecosystems (Rosa et al. 2011; Sousa et al. 2006; Oliveira et al. 2015). The lack of production of the mucilaginous drogue line by the clams in some populations renders them an incapacity of spreading through their normal rates, possibly leading to earlier mortality and/or to the extension of the expected lag time phase (Rosa, Pereira, et al. 2014).

Alterations in abiotic conditions through clam's mass mortalities, production of pseudofaeces, sediment load with shells, filtration activity, bioturbation or burrowing are a concern in the sense that these events can induce profound changes in an ecosystem, affecting the physical structure of the habitat, the sediment chemistry, organic content, oxygen availability, food chains, nutrients dynamics, redox potential, particles size and water clarity. Therefore, changes in existing biological communities are naturally expected, since coexisting species may not support life under the new habitat conditions induced by clams (Sousa et al. 2008; Ferreira-Rodríguez et al. 2016; Crespo et al. 2017; Ilarri et al. 2014; Sousa et al. 2007).

C. fluminea is generally tolerant to a wide range of environmental conditions, such as salinity and temperature. Regarding high levels of salinity, freshwater bivalves trigger their osmoregulation mechanisms to cope with this alteration, following the primary defence by the closure of their valve (Ferreira-Rodríguez & Pardo 2016). In a limiting situation, the Asian clam is no longer capable of maintaining the osmotic balance and starts outflowing water, ultimately leading to their withering.

Temperature is determinant to an adequate biological metabolism, especially in poikilothermic ectotherm bivalves such as the Asian clam. Indeed, temperature affects physical and biological aspects of the organisms, namely reproduction, feeding, shell length and body mass, and can also be a stressor against drifting and swimming (Rosa et al. 2012). Still, high temperatures might also help the clams' expansion in aquatic systems (Rosa et al. 2012).

Oxygen levels, on the other hand, can be an opportunity to develop improved control strategies. The Asian clam is very sensitive to changes in the oxygen concentrations and this is clearly visible throughout the warm seasons, as the combination of nutrient loads and high temperatures result normally in decreased oxygen concentrations and may cause massive die-offs (Mackie & Claudi 2010). Thereby, hypoxia and anoxia scenarios represent effective measures to control these biofoulers, either singly or in combination with chemical control agents (Rosa et al. 2015). Although this could be feasible in industrial facilities, it is worth noting that achieving complete oxygen deprivation in a natural ecosystem is a very difficult task, not to mention unsustainable risks for the remaining fauna and flora.

Chemical treatments are the most straightforward solution used to control the macrofouling by invading bivalves, as they are an easy, versatile and cost-effective approach that enables total protection of industrial systems. Chemical cleaning and acid mixtures designed to dissolved clam shells are two possible strategies, but the usual choice falls on the use of biocides (Sousa et al. 2014). There are already some candidate chemical compounds that have been tested in this context. For instance, potassium chloride, aluminium sulphate and polyDADMAC showed efficient molluscicidal activities, and their interest also comes from the use of aluminium sulphate and polyDADMAC in disinfection processes in wastewater treatment plants (Gomes et al. 2014). Also, the possibility of developing a biocide capable of interfering with the chemosensorial system of the Asian clam could present another challenge in what concerns target-treatments to this invasive pest, especially in water-dependent industries (Castro et al. 2018). Despite their inherent risks for the environment, yet manageable under industrial circumstances, the application of biocides in open waters needs a closer investigation in terms of their ecotoxicity to non-target species and their financial costs, along with solutions to ensure their high ecological selectivity and viability (related to the high water volumes) (Sousa et al. 2014). A review covering all methods, chemical and non-chemical, suitable to be applied in industries and open waters is presented by Mackie & Claudi (Mackie & Claudi 2010).

In summary, and despite several possibilities proposed through the years for the control of the Asian clam in fouled settings, the fact is that their efficiency is not high, and their success is generally limited by the continuous infesting inflow. In this way, it is worth exploring management strategies that can offset the clam's nuisance potential; for instance, by using the clam as a bioremediation agent.

1.4. Objectives and structure of the dissertation

The overall research question behind the present dissertation is whether the Asian clam can be used as a bioremediation agent targeted at OOMW, following on previous research denoting its capacities as a biofilter of eutrophic waters (Cohen et al. 1984); metal-loaded effluents (Rosa, Costa, et al. 2014); winery wastewaters (Ferreira et al. 2017); pesticides (Cooper & Bidwell 2006; Jacomini et al. 2006); polycyclic aromatic hydrocarbons (Silva et al. 2016); pharmaceuticals (Aguirre-Martínez et al. 2018); and microorganisms (Ismail et al. 2015; J. F. Gomes et al. 2018). In this way, three major aims were set for the enclosed studies:

(i) To critically revise the methods currently used to treat OOMW, their efficiency and the suitability/opportunities of developing new (biological) methods based on the use of the Asian clam.

(ii) To assess the filtration capacity of *C. fluminea* over OOMW.

(iii) To evaluate a possible application of *C. fluminea* shells as a biosorbent for the treatment of OOMW.

Sequentially covering these established aims, after the present general Introduction (Chapter 1), a Review chapter is presented (Chapter 2). Therein, OOMW production worldwide, composition, environmental hazardous potential, as well as treatment solutions that have been applied along with the most promising avenues for further developments in this context are detailed. Then, Chapter 3 and 4 address the bioremediation potential of *C. fluminea* evaluated in two distinct assays: a biofiltration study with living organisms (Chapter 3); and an experiment focused on the biosorption properties of the shell matrix (Chapter 4). Finally, the main conclusions and perspectives for future studies are presented as final remarks (Chapter 5).

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CHAPTER 2. The cross-talk between bioremediation and valuation of residues of the olive-oil production chain

Abstract

Olive oil mill wastewaters (OOMW) are amongst the most concerning contaminants, particularly in the Mediterranean region. Several treatments have been proposed to optimize the OOMW removal from aquatic matrices. The most conventional methods used are physicochemical processes, but bioremediation has been arising as an environmentally friendlier alternative. Biofiltration is less explored, and only a limited number of biofilters have been proposed. The integration of both approaches allows an improvement in both discoloration, COD removal and detoxification of OOMW, ensuring better results than the application of these technologies isolated. Biosorption has also been gaining attention as a surrogate approach to treat effluents, with advantages over conventional methods. The valuation of residues and by-products of the olive-oil production chain has been highlighted as a solution, stimulating the extraction of a panoply of compounds. In this chapter, the state of the art on bioremediation and biosorption approaches applied to OOMW is revisited, thus overviewing the current and proposed strategies to improve its management practices. The development of these processes and new ideas to improve already existing solutions are crucial to mitigate the environmental impacts caused by these wastewaters while valuing the by-products resulting from the olive-oil production industry, towards a circular economy approach.

Keywords: Olive oil mill wastewaters; biosorption; biofiltration; organic compounds; water quality; aquatic systems

2.1. Introduction

Earth has been experiencing profound changes through the last centuries. Since Industrial Revolution in the 18th century, Humanity continues to witness great and fast technological development, in parallel with a substantial improvement in their lifespan and living standards. Consequently, the world population has been increasing exponentially in the last years and the subsequent environmental impacts are becoming each day more evident. Agricultural and industrial activities represent major routes of pollution that are threatening the normal functioning and structure of ecosystems, since some biological and chemical processes required to maintain and preserve life have already overcome their resilience capacity (Rockström et al. 2009), and so its urgent to develop solutions and more sustainable practices to mitigate these problems and to restore the multifunctionality of ecosystems.

The Mediterranean region is known for being a region with great production of olive oil mainly due to the climatic conditions of this geographical area: summers are warm and dry, with average temperatures of 30°C, and winters are characterized as moderate due to intense and frequent rainfalls, mainly between October and March/April (Zampounis & Minoans 2006). Olive oil is considered the main vegetable fat in the regional gastronomy, not only because of its therapeutic properties (Obied et al. 2005) but also because of the flavour and aromas it adds to the recipes, thus having a high market value in all the Mediterranean and European countries (Zampounis & Minoans 2006). The importance of this industry to the economy of Portugal, Spain, Italy and Greece is notorious. In fact, data from the International Olive Oil Council states a contribution from these countries of about 94% of the worldwide production in 2017. Along with these European countries there are also Tunisia, Morocco, Turkey, Algeria, Argentina, Jordan and Egypt, which altogether are estimated to contribute with 29% of the total world production (IOOC 2017).

Notwithstanding, olive oil bears a huge water footprint and extremely toxic wastewaters that remain in the end of the production process (Gebreyohannes et al. 2016; Dermeche et al. 2013; El-Abbassi et al. 2012; Roig et al. 2006). The widespread use of the traditional oil extraction system in a large number of olive oil mills is the main contributor to the elevated water consumption rates registered in south European countries, along with the production of larger volumes of wastewaters when compared to more recent processes (IOOC 2015). The literature underlines that the discharge of 1 m^3 (a tonne) of olive oil mill wastewaters (OOMW) in receiving systems has the same environmental impact of about 200 m^3 of domestic wastewater (El-Abbassi et al. 2012). If one considers that 1 tonne of olives generates between 1 and 2 tonnes of OOMW, and that the Mediterranean countries discharge 30 million m^3 of this effluent each year, the magnitude of the consequences associated with this practice becomes clearly anticipated (Gebreyohannes et al. 2016; Jalilnejad et al. 2011). Facing these numbers, it is urgent to design solutions not only to reduce the volume of OOMW but also to treat this externality. The present chapter will focus on the current strategies to improve OOMW treatment and management practices targeted at these effluents, as well as, it aims to propose new avenues that can be further explored in this context.

2.1.1. Regulation for OOMW discharges in South European countries

Once recognized the environmental impacts of OOMW, there have been increasing efforts to regulate OOMW discharges in South European countries. Thus, particularly focused in the safety and protection of natural resources, *vis.* water and soils, several policy frameworks have been developed and enforced through the years to mitigate the ecological impacts of this anthropic pressure, in terms of ecosystems structure, function and services.

In Europe, the Water Framework Directive (WFD) is focused in the monitoring and maintenance of the ecological status of aquatic systems (surface, transition, coastal and groundwaters) with the aim of promoting all water bodies to a good ecological status by 2027 (Council of the European Communities 2000). To achieve this, several measures must be encouraged, including sustainable water consumption; a reduction in discharges, emissions and/or losses of priority substances to the aquatic environment; and protection of marine waters. Albeit the enforcement of this legislation triggered some environmental responsibilities to the EU member states, regarding the continuous monitoring of their waters by the competent entities, there are still gaps in the regulations that allow mismanagement. For example, the non-effectiveness of the "polluter-paying principle", since the fines of inappropriate wastewater disposals are more cost-efficient than the correct implementation of a water treatment facility, at least for small/medium industries.

Focusing on the disposal of olive oil effluents, the demands vary from no legislation regarding OOMW discharge into the natural environment in Greece (Azbar et al. 2004; Inglezakis et al. 2012; Kapellakis et al. 2008) to the more firm legislation of Spain, where it is strictly forbidden to discharge untreated OOMW in natural systems (Azbar et al. 2004; Inglezakis et al. 2012; Kapellakis et al. 2008). Also, Spain has promoted the construction of about 1000 evaporation ponds to deal with OOMW, along with the implementation of the two-phase systems to reduce OOMW volume (Azbar et al. 2004; Inglezakis et al. 2012; Kapellakis et al. 2008). Both Italy and Portugal have softer legislation compared to Spain. Italy allows the spread of treated OOMW to agricultural lands under controlled conditions after notification of the competent authorities (Azbar et al. 2004; Inglezakis et al. 2012; Kapellakis et al. 2008). In Portugal, olive oil mills must mandatorily establish contracts with water and sanitation authorities to deal with produced OOMW, along with the implementation of preventive measures for accidental discharges (Normative Order no. 118/2000; Ordinance no. 1030/98). Wastewater discharges in waters and soils are allowed, as long as olive oil producers require a licence and fulfil the criteria established in the Normative Order no. 626/2000, and more generally in the Law-by-Decree no. 236/98.

Despite the development of environmental policies aimed at mitigating the hazardous potential of OOMW, it is evident that its requirements and enforcement have not been successful since impacts in aquatic and terrestrial ecosystems continue to be reported worldwide (Elhag et al. 2017; Pavlidou et al. 2014; Karaouzas et al. 2011; Aharonov-Nadborny et al. 2018; Sierra et al. 2001). A consensual European framework should be developed with the intent of unifying and disseminating the best environmental practises supported by all the evidences generated by the scientific community (Koutsos et al. 2018) – for instance, protective European frameworks directed to both soils and wastewaters are urgent and crucial in the case of OOMW (Komnitsas & Zaharaki 2012). On the other hand, through the development of sustainable solutions promoting the re-use of OOMW in activities such as agriculture, circular economy principles can apply by transforming a waste into a valuable product to producers, while a highly concerning environmental problem is being solved. The existence of financial incentives to support the implementation of infrastructures for treatment, storage, distribution and disposal of OOMW is crucial for the successful commitment of the producers. With a common regulation, addressing three main topics: treatment, management practises and practical applications, along with the definition of maximum allowed benchmark value for discharge to be adopted by all EU member states, policy makers will more easily frame the optimization of olive oil mills, ultimately satisfying the social demands towards more sustainable production processes (Koutsos et al. 2018).

2.2. Olive Oil Mill Wastewaters (OOMW)

2.2.1. Olive oil production processes

Olea europaea L., commonly known as olive, is the species that supports the olive oil value chain. Olive oil extraction is a very well-tuned process regarding the adequate treatment and possible valorisation strategies of solid wastes (olive husk or pomace) and effluents (Roig et al. 2006; Morillo et al. 2009). The production of olive oil is commonly conducted between November and February and it is achieved either by a continuous centrifugation (modern process) or by the application of discontinuous pressure in the olives (oldest and most traditional process).

In general, the traditional process involves the following sequential steps: (1) crushing, (2) pressing, and (3) decantation. After crushing the olives, the resulting paste is mixed and homogenized to aggregate all oil droplets. The paste is then placed in the hydraulic press with the intention of squeezing it as much as possible - a small amount of water is added in this step to help the separation of the oil/water phases. The remaining product is a mixture of olive pulp, peel, stone and water, generally defined as olive pomace. The water mixed with olive oil is later separated by decantation. At the end of this process, 3 components remain: olive oil, pomace and OOMW. Although this is a simple method involving inexpensive equipment and producing low amount of OOMW, it is a discontinuous process entailing high energy costs. In addition, the OOMW obtained represents high rates of (bio)chemical oxygen demand (BOD) in receiving environmental compartments (Dermeche et al. 2013), denoting a relevant input of organic contaminants.

The continuous process comprises either a three-phase (3P) or a two-phase system (2P), and the main difference between them is the final product. The 3P system yields three final products: olive pomace (solid phase), OOMW and olive oil (liquid phases). The main problem with this system is the enormous amount of water necessary for the centrifugation step that is directly related to the final amount of OOMW produced. The 2P system was developed to minimize the volume of OOMW, thus there is a single addition of water in the crushing phase. In the 2P system there are only two remaining products: olive oil and wet pomace, commonly designated by TPOMW (“two-phase olive-mill waste”), which is a mixture of OOMW and olive husk that can be reprocessed to enhance the extraction yield (Dermeche et al. 2013; Aggoun et al. 2016). Figure 2.1 shows the steps of each production processes of olive oil.

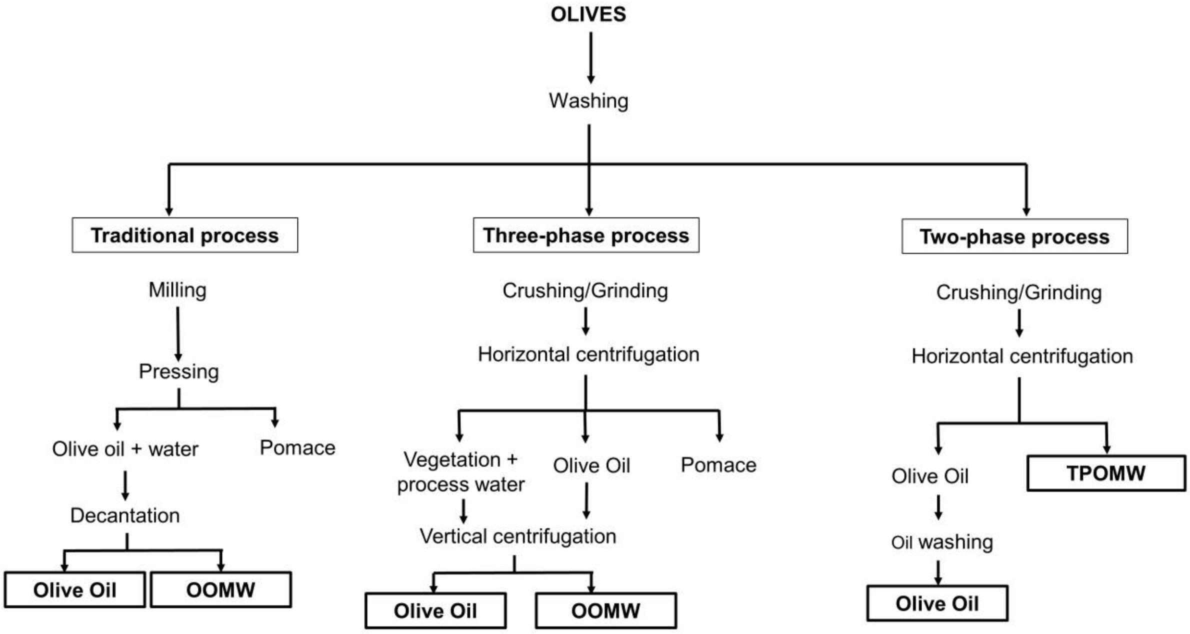

Figure 2.1. Production processes for olive oil (adapted from Morillo et al. 2009).

2.2.2. Characterisation, physical and chemical properties of OOMW

OOMW properties depend on olive orchards management, olives maturation and extraction processes, as well as on the storage time and regional climatic conditions (McNamara et al. 2008; Aggoun et al. 2016; Justino et al. 2010).

OOMW is known to have a dark colour and an intense smell (McNamara et al. 2008; Justino et al. 2010). The main properties of this wastewater are a highly acidic pH, a high turbidity, a high conductivity, a high content of suspended solids and a high biochemical and chemical oxygen demand (COD and BOD, respectively). BOD typically ranges from 35 to 110 g/L, whereas COD ranges between 40 and 220 g/L (Cassano et al. 2013; Dermeche et al. 2013). These properties can be explained by the high organic content of OOMW, such as lipids, sugars (fructose, mannose, glucose, sucrose, pentose and sucrose), tannins, pectins, polyalcohols and polyphenols, along with some inorganic salts (namely potassium, calcium, sodium, magnesium and iron).

Polyphenols are the major components of OOMW in concentrations of about tens g/L, particularly hydroxytyrosol, tyrosol and oleuropein (Figure 2.2) (Justino et al. 2010; Justino et al. 2012; McNamara et al. 2008; Cassano et al. 2013). Although these compounds have potential benefits to human health, their presence is usually not taken into account in what regards olive oil nutritional quality (Gebreyohannes et al. 2016).

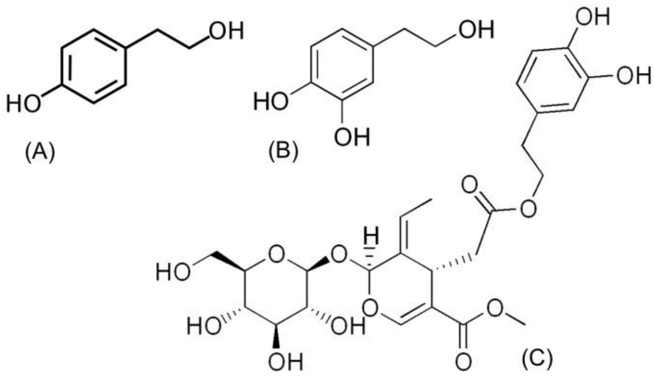

Figure 2.2. Chemical structures of the three most important polyphenols in OOMW: A – tyrosol; B – hydroxytyrosol; C – oleuropein.

Other phenolic compounds present in OOMW, such as flavonoids and carotenoids, have great potential to benefit human health and well-being due to their high activity as antioxidants, anti-inflammatories, antiallergics, antivirals and antitumor compounds (Aggoun et al. 2016; El-Abbassi et al. 2012; Obied et al. 2005). Amongst them, hydroxytyrosol is the one with greater antioxidant activity given its great ability to protect cells against oxidative stress (Aggoun et al. 2016; Kalogerakis et al. 2013; El-Abbassi et al. 2012). Antioxidant compounds act through the chelation or scavenging of free radicals, such as the superoxide anion, hydrogen peroxide or the hydroxyl radical preventing them to impair biomolecules such as proteins, lipids and DNA. According to El-Abbassi et al. (El-Abbassi et al. 2012), the antioxidant activity of OOMW can be directly related to the amount of free hydroxytyrosol, potentially enhanced by the presence of inorganic salts.

OOMW is different from the other oil extraction by-products, TPOMW and the pomace. OOMW is mainly composed by water and by the organic and inorganic compounds as mentioned above. TPOMW contains essentially olive husk, OOMW and inorganic salts; in the OOMW fraction tyrosol, hydroxytyrosol, p-coumaric and vanillic acids can also be found, apart from water. TPOMW is a very dense fluid because, it is highly concentrated and with a reduced water percentage. The olive pomace includes cellulose, hemicellulose, lignin, fats and proteins. The main polyphenols existing in the pomace are salidroside, nuezhenide and nuezhenide-oleoside, all of them with more complex chemical structures compared to those in OOMW (Dermeche et al. 2013).

Olive oil wastes hold relevant microbial communities. The bacterial communities present in OOMW and TPOMW are mostly composed by *Alphaproteobacteria,* *Betaproteobacteria, Gammaproteobacteria, Firmicutes and Actinobacteria*. Relatively to fungi, *Candida spp, Pichia* and *Sacharomyces* are the most common yeast taxa found in OOMW; whilst in TPOMW the predominant biota is composed by *Pichia* and *Sacharomyces*. Acidic conditions and high concentrations of salts and sugars favours the growth of osmotolerant yeasts and bacteria, and this could be explained by the higher ability of fungi to decompose organic matter, or simply by a better adaptation of yeast to the conditions of OOMW as a growth medium (Ntougias et al. 2013; Ben Sassi et al. 2006).

Finally, it is worth noting that the storage of OOMW induces some chemical modifications. Olive oil undergoes lipids oxidation and hydrolysis, with the subsequent degradation of its quality caused by the by-products of these reactions. The quality of the olive oil directly relates to its physical-chemical properties, so the non-filtered olive oil is more unstable due to the presence of suspended solids. The characteristic dark colour of OOMW is also a result of the previous degradation routes that adversely affect the antioxidant activity of the polyphenols, thus promoting the recalcitrant capacity of this wastewater (Kalogerakis et al. 2013).

Volatile compounds are mainly hydroperoxide anions, their production augmenting in the presence of light, metals, high temperature, pigments, unsaturated fatty acid, but also depending on the quantity of natural sterols and antioxidants (Angerosa et al. 2004). These parameters, along with the presence of microorganisms, originate the typical strong smell of stored OOMW (Dermeche et al. 2013; Justino et al. 2009). In a study about the volatilization of OOMW compounds after its spreading in soils (Rana et al. 2003), the authors concluded that OOMW emissions are mainly composed by phenol compounds and sulphur dioxide, and that these emissions are more likely to occur with higher temperatures.

2.2.3. Environmental hazardous potential of OOMW

The environmental hazards of chemical compounds are typically evaluated according to, three phenomena: bioaccumulation, persistence and toxicity. A surrogate commonly used for bioaccumulation is the oil/water partition coefficient (K_{ow}), which evaluates the ease of a chemical to associate with an organic or aqueous phase – when this ratio is higher than 5, the focused compound is lipophilic. Persistence is defined by the ability of a chemical to withstand environmental degradation and thus succeed to be transported over long distances with respect to its original disposal site. The persistence of a given compound can be ranged according to its half-life in water (hydrolysis), sediment or soil, and can be mediated by certain factors such as light (photolysis) or microbial communities (biodegradation). Finally, toxicity evaluates the ability of a given dose of a chemical (parent compound or degradation product) in inducing any type of adverse effects in the biota (Muir & Howard 2006; Wania 2003), clarifying cause-effect relationships.

Polyphenols are the most concerning compounds in OOMW, as they bioaccumulate and induce several toxic effects. Polyphenols tend to be dominant in the wastewater, according to, their partition coefficient established between 6×10^{-4} to 4 (Obied et al. 2005; Aggoun et al. 2016). In addition, this parameter is influenced by temperature and by the water volume added along the olive oil extraction process: under higher temperature, the partition of polyphenols into the oil phase is promoted; if the water volume is increased, they are preferably transferred into the aqueous phase, i.e. the OOMW (Obied et al. 2005). Studies focusing on the persistency of OOMW are scarce and the half-life of its phenolic compounds either in soils or in water hasn't been determined yet. On the other hand, phenolic compounds are the main responsible for the toxicity attributed to the olive oil wastewaters. The hazard potential associated with phenolics has been related to a high ability in inducing the production of reactive oxygen species (ROS), narcosis and respiratory disturbances (Justino et al. 2012).

It has been proven that the most toxic OOMW is the one resulting from the traditional process as compared with the 3P and 2P extraction processes (Ben Sassi et al. 2006). This can be explained by the fact that the effluent resulting from traditional treatment has the lowest water content, which renders a higher concentration of phenolic compounds compared to effluent yield from the other processes (Ben Sassi et al. 2006). Despite the process, OOMW exhibit notorious toxic effects in the biota of water and soil compartments provided their common discharge directly into rivers or in agricultural soils for irrigation purposes (Fiorentino et al. 2003; Andreozzi et al. 2008; Isidori et al. 2004; I. Karaouzas, E. Cotou, T.A. Albanis, A. Kamarianos, Nikolaos T. Skoulikidis 2010; Venieri et al. 2010). Moreover, following deposition in soils, these wastes may leach to groundwaters, triggering a contamination cycle that may also contribute to additionally impact aquatic ecosystems.

Fiorentino et al. (2003) exposed four freshwater organisms *(Pseudokirchneriella subcapitata, Daphnia magna, Brachionus calcyflorus* and *Thamnocephalus platyurus)* to fifteen phenolic compounds highly abundant in OOMW (including tyrosol and hydroxytyrosol), and all compounds exhibited toxic potential to one or more organisms. Growth inhibition and mortality were the main toxic effects reported in several aquatic species after exposure to OOMW including *P. subcapitata* (Andreozzi et al. 2008), *D. magna* (Isidori et al. 2004), *D. longispina* (Justino et al. 2009), *Gammarus pulex* (Karaouzas et al. 2011b), *Hydropsyche peristerica* (Karaouzas et al. 2011b) and *Danio rerio* (Venieri et al. 2010).

Beyond the impacts registered directly in organisms, the discharge of OOMW into the environment may transform clear and transparent waters into an opaque and highly contaminated system, a scenario that may translate into severe impacts on aquatic and terrestrial ecosystems. One of the most common downstream environmental problems resulting from OOMW discharge is eutrophication in rivers and lakes, primarily because these effluents contain high concentrations of nutrients, thus promoting the overgrowth of aquatic microalgae and plants. OOMW has a high amount of sugars in its composition that induce microbial metabolism, as well as stimulate the growth of aquatic flora (McNamara et al. 2008). Moreover, lipids and polyphenols composing the OOMW could block the entrance of sunlight and oxygen in the aquatic systems due to the formation of a biofilm at the water surface, thus directly constraining the growth of water column and submerged producers and also compromising oxygen levels available for the remaining biota (McNamara et al. 2008; Dermeche et al. 2013). Eutrophication does not only deteriorate the ecological and chemical status of natural ecosystems but may also deteriorates recreational and touristic activities, fishing and sailing, raising economic and cultural concerns.

Discharge of OOMW in soils leads to the impairment of the soil structure by changing the soil resistance and increasing its salinity levels. In addition, phenolic compounds trigger toxic effects and have the potential of inhibiting the germination and growth of plants (Barbera et al. 2013; Karpouzas et al. 2010; Chatzistathis & Koutsos 2017; Dermeche et al. 2013), as well as the reproduction of some invertebrates (Hentati et al. 2016). Regarding adverse effects in soil species, plants growth impairment and seeds germination inhibition were the most commonly observed effects in terrestrial species with agronomic importance as *Latuca sativa* (Priac et al. 2017), H*ordeum vulgare* (Ben Sassi et al. 2006), *Lepidium sativum* (Tsioulpas et al. 2002), *Raphanus sativus and Cucumis sativus* (Andreozzi et al. 2008).

Microorganisms like *Bacillus subtilis*, *B. megaterium, Escherichia coli*, *Pseudomonas aeruginosa, P. fluorescens, Staphylococcus aureus*, *Streptococcus pyogenes*, *Klebsiella pneumoniae* and *Vibrio fischeri* were all found to be negatively affected by exposure to OOMW through the inhibition of growth rates and luminescence (Obied et al. 2007; Mekki et al. 2008; Azaizeh et al. 2011; Dhouib et al. 2006). Shifts in the physiological profile of microbial communities were also visible after the exposure of soils to high polyphenols concentrations (Hentati et al. 2016), which lead to modifications in the local microflora diversity.

2.3. OOMW management

2.3.1. OOMW valuation strategies

Valuation can be defined as a strategy that aims to attain a sustainable development through the management of industrial by-products, wastes and wastewaters until their ultimate reuse either for industrial processes or agriculture, or through the recovery of its fine chemicals (El-Abbassi et al. 2017). The Mediterranean region has the most significant olive oil production worldwide, which means that it also possesses the major volumes of OOMW to deal with. This region is characterized by arid soils, water scarcity and low energy resources, thus it is imperative to develop and apply proper management strategies based on reuse and treatment of OOMW to achieve economic and environmental sustainability. The valuation of OOMW can be done by using it for different applications, or by taking profit of some of their components.

Application of olive oil by-products in soils as substitutes of chemical fertilisers has been a hot-topic within the scientific community in order to simultaneously value these wastes and to overcome their environmental damage (El-Abbassi et al. 2017; Chatzistathis & Koutsos 2017). Research in this field is challenging and several avenues are worth exploring in its context. For example, application of OOMW in olive orchards might be a viable management solution for this effluent, and this was the basis of the work by Mechri et al. (2009). Their focus was on the putative consequences of the application of this waste as a fertiliser in the olive trees development, in the olive fruit and, at last, in the olive oil quality. Their findings suggest that arbuscular mycorrhizal fungi were negatively affected by OOMW, reducing the colonization of the olive tree roots. This process gradually brought other side effects contrasting to the increase of olive oil phenol load: reduction of the photosynthetic rates and in nitrogen, phosphorus and oil contents of the olive fruit. In fact, the main conclusion of this study was that all these parameters have a huge importance in the final characteristics of the olive fruit, which by itself controls the overall olive oil quality.

Generally, the application of OOMW in field crops should always be carefully controlled for volumes since the spreading of a larger volume could lead to profound changes in soil structure and properties, thus constraining the production outcome (Barbera et al. 2013). This control should not be decided based only on the polyphenol content of OOMW, but must also take into account a series of other variables such as soil type, pH, electrical conductivity, total organic matter, total nitrogen and nitrates, available phosphorus, exchangeable potassium and soil moisture; the toxic effects of OOMW are a result of a complex interaction between all these variables and not the direct consequence of the increase in polyphenols (Hentati et al. 2016; Komnitsas & Zaharaki 2012). A long- and short-term assessment of the impacts of spreading OOMW in soils was presented by Di Bene et al. (2013). They concluded that soils tend to reach their initial chemical state after being in contact with OOMW, although recognising the risk of applying these effluents in terrestrial systems. Nevertheless, OOMW bears some important benefits to enriched soils, namely: improvement in porosity and aggregate's stability; minimization of runoffs (and a consequent increase of water retention capacity) and erosion; enhancement of soil organic matter; temporary carbon enrichment as well as that of other nutrients, which enhances soil fertility and may reduce the need of fertilizers; stimulation of microflora development; inhibition of microorganisms; and as a control agent against phytopathogens, mainly due to the antimicrobial compounds present in these wastewaters (Barbera et al. 2013; Karpouzas et al. 2010; Chatzistathis & Koutsos 2017).

Composting is another solution for OOMW that relies essentially in the value of biomass as a natural resource with several environmental benefits: highly abundant, renewable, preserves carbon dioxide atmospheric balance, contains small amounts of nitrogen and sulphur, and it's an important energetic alternative in the future due to its low footprint and human health impacts (Hernández et al. 2014). Composting is an aerobic process that takes advantage on the high metabolic efficiency of microorganisms that are present in the OOMW. It has three key stages: (1) activation phase, that presumes the stabilization of humidity and aeration conditions; (2) thermophilic phase, where there is an increase in the reaction temperature that promotes the activity of bacteria and fungi and the (3) mesophilic phase, where the biomass reaches the surrounding temperature. At the end of the process, carbon dioxide and humus remain, the latter also called compost, with large potential either as a soil amender or as a fertiliser (Muktadirul Bari Chowdhury et al. 2013). The compost is mostly composed by organic matter, nitrogen, phosphorus and trace elements. Humic substances result from the degradation of large organic compound chains, like cellulose, hemicellulose and proteins, which are also main components of olive oil pomace (Tortosa et al. 2014). All these compounds are crucial to the structure and fertility of soils by affecting e.g. water retention capacity, cationic exchange capacity, microbiologic activity and degradation of pesticide residues; and also for plants physiology, nutrient uptake and root development (Muktadirul Bari Chowdhury et al. 2013;

Tortosa et al. 2014). The soil microbiota may greatly benefit with both OOMW and olive oil compost input, and this can translate into the increase in total population diversity (bacteria and yeasts), along with enhancements in extracellular enzymatic activities and in functional and catabolic activities (Muktadirul Bari Chowdhury et al. 2013). In particular, compost foments the proliferation of specific bacterial communities and microbial consortia specialized in the degradation of organic compounds (polyphenols, tannins and lipids), which further enhances OOMW importance in soils bioremediation (Ntougias et al. 2013). OOMW can be composted alone or mixed with other agro-residues, which is an advantage for small olive oil mill owners (Chowdhury et al. 2014; Aquilanti et al. 2014). The benefits of this compost in agriculture, along with the production of large volumes of olive oil wastes and the reduced organic matter contents of the Mediterranean soils, may render composting a powerful sustainable solution for OOMW (Ochando-Pulido et al. 2017).

Biosorption can play a major role in the olive-oil production chain, namely by using the solid residues in the treatment of contaminated aqueous solutions. In fact, this is a widely studied solution (Table 1) that has great potential: these waste matrices have strong adsorption properties; they are a low-cost solution, generally produced at a local level and in large amounts. The main solid residues that result from the olive oil production are: (1) olive stones, which refer to the olive pits, cores or kernels; (2) pruning material, which includes the woody wastes of the olive trees such as the branches and twigs; (3) olive leaves from the trees resulting from the de-leafing of olives before washing; (4) pomaces already described in section 1.2.1; and (5) exhausted pomaces, also called exhausted cakes, that result from pomaces that have been further processed to extract the remaining oil that may exist (Anastopoulos et al. 2015). Each one of these residues has its own physicochemical characteristics that confer them unique biosorption properties. Moreover, the kind of activation (physical or chemical) used and the processing conditions are other key determinants of the sorbent capacity of olive solid wastes (Bhatnagar et al. 2014). There are already a couple of reviews on the sorption characteristics of these residues, as well as on the different treatment methods and other conditions that may be applied to improve yields (Bhatnagar et al. 2014; Anastopoulos et al. 2015). Therefore, here we will mostly summarise the suitability of each type of waste to treat aqueous solutions contaminated with specific sorbates and update on the most recent advances that were not covered in previous reviews.

Olive stones (i.e. olive pits, cores or kernels) seems to be the most widely explored by-products as biosorbents (Anastopoulos et al. 2015; references within Table 1). This is probably due to the fact that these wastes are highly lignified, rendering them an advantageous source to produce activated carbon (Anastopoulos et al. 2015). Olive stones were proven to successfully adsorb some metals (Al, Cd, Cr, Cu, Fe, Ni, Pb, U, Zn) and dyes (Alizarin Red, Methylene Blue, Safranine, Remazol Red B), as well as the pesticide aldrin, the pharmaceutical amoxicillin and phenols (Table 1).

On the other hand, olive pruning and leaves have been suggested as sorbents of metals (Cd, Cu, Ni, Pb; Table 1), but they are the least studied wastes in this context despite being amongst the major wastes of the olive-oil production chain. This incongruence is likely to be due to difficulties of collection, handling and shredding prior to use (Anastopoulos et al. 2015). Pomace has been suggested as a successful biosorbent for several metals and related elements (Cd, Cu, Cr, Fe, Hg, Ni, Pb, U, Th, Zn), dyes (Safranine, Remazol Red B, Reactive Blue 19, Reactive Red 198), pesticides (aldrin, endrin, dieldrin), radioisotopes (Ga-67, Tl-201, Cs-137) and phenols (Table 1). Still, the potential of olive pomace can be extended further since it can be used as an inoculum of hydrocarbonoclastic bacteria, which are useful for hydrocarbon-bioremediation, e.g. in cases of olive oil spill (Dashti et al. 2015). The exhausted cake has been suggested as a sorbent of different metals (Cd, Cr, CrO_4^{2-}, Fe, Ni, Pb, Zn), dyes (Lanaset Grey G, Black Dycem TTO), and pesticides (dimethoate, imidacloprid, diuron, tebuconazole and oxyfluorfen). More recently, some studies started to arise combining different debris to increase the potential of the byproducts to treat contaminated solutions. For example, Delgado-Moreno et al. (2017b) proposed the use of wet olive cake and vermicompost, together with olive tree pruning, as substitutes of some of the components of biomixtures used in biobed purification systems to adsorb pesticides.

The valuation of residues of the olive-oil production chain should be a priority given the innumerous problems associated with their disposal. Moreover, considering the increasingly serious environmental contamination scenarios worldwide, the possibility of using these wastes to biosorb some problematic sorbates is something that is worth to explore.

Table 1 - Contaminants reported to be biosorbed by the solid residues of the olive-oil production chain. Information on studies published before 2014 was retrieved from the review by Anastopoulos et al. (2015). The residues considered in this summary include derivatives such as ashes.

Residue	Contaminant				References
	Metals	Dyes	Pesticides	Others	
Olive stones	Al Cd Cr Cu Fe Ni Pb U Zn	Alizarin Red Methylene blue Safranine Remazol Red B	Aldrin	Pharmaceuticals (amoxicillin) Phenols	(Anastopoulos et al. 2015; Albadarin & Mangwandi 2015; Calero et al. 2016; Calero et al. 2018; Dardouri & Sghaier 2017; Hodaifa et al. 2014; Limousy et al. 2017; Moubarik & Grimi 2015; Ronda et al. 2015; Trujillo et al. 2016)
Pruning material	Cd Cu Ni Pb	-	Dimethoate Imidacloprid Diuron Tebuconazole Oxyfluorfen	-	(Anastopoulos et al. 2015; Almendros Molina et al. 2016; Delgado-Moreno et al. 2017b; Delgado-Moreno et al. 2017a)
Olive leaves	Cd Cu Pb	-	-	-	(Anastopoulos et al. 2015)
Pomace	Cd Cu Cr Fe Hg Ni Pb U Th Zn	Safranine Remazol Red B Reactive Blue 19 Reactive Red 198	Aldrin Endrin Dieldrin	Radioisotopes (Ga-67, Tl-201, Cs-137) Phenols Spilled crude oil	Anastopoulos et al. 2015 Martinez-Garcia et al. 2006 Venegas et al. 2015 Dashti et al. 2015 Petrella et al. 2018 Kučić & Simonič 2017
Exhausted cake	Cd Cr CrO_4^{2-} Fe Ni Pb Zn	Lanaset Grey G Black Dycem TTO	Dimethoate Imidacloprid Diuron Tebuconazole Oxyfluorfen	-	Anastopoulos et al. 2015 El-Kady et al. 2016 Delgado-Moreno et al. 2017b Delgado-Moreno et al. 2017a

Further applications that significantly increase the commercial value of OOMW are the use of these wastes as biofuels - biodiesel, biohydrogen, biomethane, bioethanol (Hernández et al. 2014; Dermeche et al. 2013; Christoforou & Fokaides 2016) - and as alternative energy resources (Chouchene et al. 2012; Jeguirim et al. 2012). OOMW could also be the source of many added-value products. Single cell oils, mannitol and citric acid are examples of carbon sources that can be produced biotechnologically through the use of OOMW as a substrate for the microorganisms. (Dourou et al. 2016). Moreover, several bioactive compounds can be recovered from OOMW that are highly valuable to human health (Aggoun et al. 2016). Polyphenols have been gaining interest as bioactive compounds due to their high antioxidant potential, which makes them adequate to be applied in food, cosmetics and pharmaceuticals development, either as additives or nutrients (Obied et al. 2005; Aggoun et al. 2016). Fractionation of the existing fatty acids in the OOMW allows the recovery of oleic and linoleic acids, which may be very useful in pharmaceutical applications requiring products of high purity, such as pure glycerol and biodegradable soap (Elkacmi et al. 2017).

2.3.2. OOMW treatment processes

It is imperative to develop methods to treat OOMW to mitigate their nuisance effects, such as odour and the contamination of soils, groundwaters and superficial waters (Ioannou-Ttofa et al. 2017; Kavvadias et al. 2010; Paraskeva & Diamadopoulos 2006).

OOMW is commonly treated under a set of general premises: (1) a good treatment process must take into account the seasonality of olive oil production, the distribution and local operations, (2) the efficiency of the method, and (3) the simplicity of the method (McNamara et al. 2008). Technological processes have been developed to mitigate the nuisance potential of OOMW (mainly focused on polyphenol removal) that include physicochemical techniques, biological techniques, or a combination between them (Figure 2.3). Nevertheless, the implementation of available treatment solutions remains a challenge to small producers due to economic constraints (Ioannou-Ttofa et al. 2017).

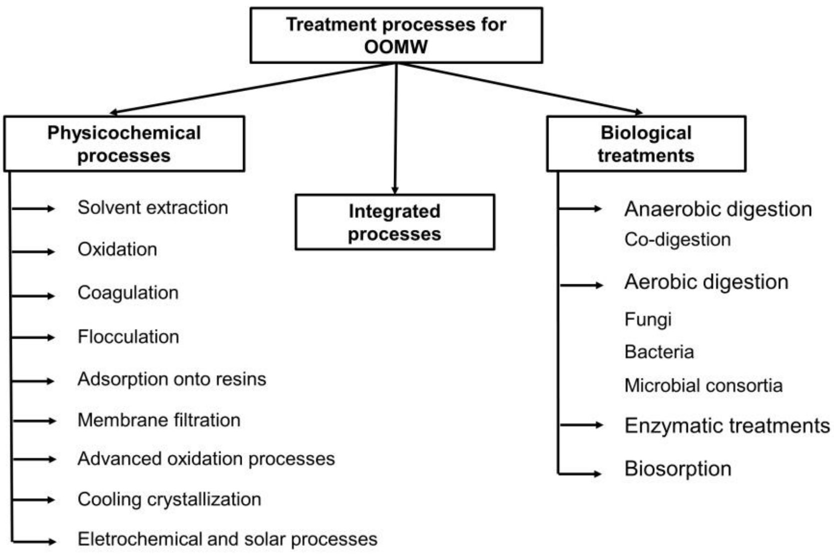

Figure 2.3. Summary of treatment processes currently described for OOMW.

2.3.2.1. Physicochemical processes

Physicochemical processes are based on solvent extraction, oxidation, coagulation, flocculation, adsorption onto resins, membrane filtration, or an integrative approach comprising several of these techniques (Ochando-Pulido et al. 2017).

Solvent extraction can be applied as liquid-liquid or solid phase extraction. Regarding liquid-liquid extraction, the most efficient solvents for polyphenols are, in decreasing order: ethyl acetate, a mixture of chloroform with isopropyl alcohol, and diethyl ether. Ethyl acetate is the most used one because it combines a potent polyphenol recovery with a low environmental footprint. This methodology is applied in many small olive oil mills mostly due to the low economic costs and the easiness of the technique, but the whole process viability is reduced as compared to other solutions (Kalogerakis et al. 2013).

Oxidation, coagulation and flocculation can be applied alone or combined, given a huge variety of treatment possibilities. Advanced oxidation processes are based on technologies that use ozonation, photolysis, photocatalysis, ultrasounds, wet air oxidation or Fenton reaction (Nogueira et al. 2016), basically for purifying waters and wastewaters by removing the organic and inorganic particles. Ozonation is widely used in wastewater treatment plants because it is an effective technique, but it is not well established that ozone is capable of reducing effluents toxicity (Justino et al. 2012). Photocatalysis is based on semiconductors coupled with either oxidants, light or both, with the intent of releasing reactive oxygen species capable of degrading both organic and inorganic contaminants (Nogueira et al. 2016). The most used oxidation process is the Fenton process due to its wider advantages: it does not generates residues; it does not needs special equipment; it's easy to be scaled up; it is highly efficient and at the same time it is not specific, which means that it can be applied to a wide range of matrices (Justino et al. 2010). The photo-Fenton reaction consists basically in the irradiation of the Fenton reagent with UV light (like solar energy, abundant in Mediterranean countries) that enhances the whole process by promoting the release of more OH• radicals (Ebrahiem et al. 2017). Although this technique appears to be a powerful tool in wastewaters treatment, its ability of nullifying their toxicity is not yet accepted, as the formation of reactive species could come along with the formation of intermediates that may pose a higher threat than their parent compounds (Justino et al. 2012; Justino et al. 2010). The application of the Fenton process is generally combined with other processes such as coagulation and flocculation (Ochando-Pulido et al. 2013; Ebrahiem et al. 2017; Ochando-Pulido et al. 2017; Ioannou-Ttofa et al. 2017).

Cooling crystallization is another ongoing alternative that separates the organic compounds present in OOMW according to their freezing point, which also allows their selective recovery at the end of the process. However, this technique is not yet fully developed allowing application at an industrial scale (Kontos et al. 2014). Electrochemical, solar and integrated physicochemical processes are also interesting solutions rising (Ochando-Pulido et al. 2017; Gursoy-Haksevenler & Arslan-Alaton 2015).

When comparing among described physicochemical processes to treat OOMW, the scientific community points out membrane filtration as the most viable solution, since it is the most efficient in fully recovering phenolic compounds. Some of the advantages of this process are the ease of implementation at the industrial level, low energy consumption, high separation efficiency, no need for additives/solvents, possibility to operate at room temperature, small area requirements and low maintenance costs (Cassano et al. 2013; Bazzarelli et al. 2016; La Scalia et al. 2017).

2.3.2.2. Biological treatments for OOMW

Biological processes that may be used to treat OOMW include anaerobic and aerobic digestion, and biosorption (already introduced in the section 3.1). Generally, biotreatments are able to remove organic and inorganic particles and have a more sustainable, environmentally friendly and reliable nature, as well as better cost-efficiency ratios compared to physicochemical approaches (Paraskeva & Diamadopoulos 2006; Daâssi et al. 2014). A review covering the advantages and problems of aerobic and anaerobic bioreactors was presented by McNamara et al. (2008).

Anaerobic digestion is an eco-friendly treatment process that offers many advantages: low production of nutrients, wastes, greenhouse gases and the production of both fertilizers and biogas (Sampaio et al. 2011). OOMW consists in a highly recalcitrant solution that cannot be directly subjected to anaerobiosis due to its high toxicity to methanogenic consortia (Hamdi 1992) – most of the times this treatment demands dilution, nutrient input or pH adjustment to prevent microorganisms growth inhibition. On the one hand, the application of these pre-treatments may pose some alterations in the organic composition of the wastewater, which decreases its energetic potential and increases the operation costs; on the other hand, it may dilute the OOMW to reduce phenolics' concentration at the expenses of an increment of wastewater volumes for further treatment (McNamara et al. 2008; Paraskeva & Diamadopoulos 2006). Alternatives based on complementary effluents have been exploited, namely co-digestion. This approach basically consists in the mixture of two different effluents, allowing the reduction of COD in the overall treatment process, rendering a better cost-efficiency ratio and enabling optimised conditions (e.g. pH and nutrients) for microorganisms. Moreover, the combination of OOMW with annually produced effluents enhances the viability of this process. There are several possible effluents that may apply to co-digest OOMW, as piggery, household waste/sewage sludge, abattoir, municipal wastewater and manure (Sampaio et al. 2011; Paraskeva & Diamadopoulos 2006).

Aerobic treatments may also apply to the degradation of phenolics in OOMW. Studies using fungi (*Geotrichum candidum, Candida boidinii, Penicillium sp, Aspergillus niger, Trametes versicolor)* claim that the most efficient removal yields regarding colour, COD and phenolic concentrations were obtained when these strains were gradually exposed to the wastewater, as this stimulated their metabolism to increase their levels of detoxifying enzymes (Assas et al. 2002; Aissam et al. 2007; Ergül et al. 2009; Fadil et al. 2003). Nonetheless, studies that do not include this adaptation step also reached good treatment results regarding OOMW (Daâssi et al. 2014; García García et al. 2000; Karakaya et al. 2012).

Laccase belongs to a group of enzymes capable of degrading aromatic compounds known as ligninolytic enzymes – these are produced extracellularly and include manganese and lignin peroxidases, amongst others. The oxidation ability of this enzymes towards high and low molecular weight polyphenols makes them biotechnologically valuable, and the microorganisms that produce them an efficient environmental solution, not only for OOMW but for other effluents too. As the aromatic compounds are quite similar to lignin monomers in terms of structure, and fungi are the main degraders of ligninolytic compounds, it is straightforward that fungi are considered the suitable and powerful microorganisms to treat matrices contaminated with these type of chemicals (Duarte et al. 2014). In addition, the residue that remains after enzymatic degradation could still be processed to further obtain added-value products (Zerva et al. 2017).

Pleurotus spp. is a widely studied genus regarding OOMW treatment (Aggelis et al. 2003). Experiments with *P. ostreatus* showed this fungus' ability to grow on sterilized OOMW and to reduce its toxicity towards *Artemia* sp. and towards seeds of *L. sativum* (Aggelis et al. 2003). Despite that, the detoxifying action promoted by *P. ostreatus* laccase enzymes generates reaction products with higher toxicity than the initial ones. Tsioulpas et al. (2002) added evidences on the ability of *Pleurotus spp* to remove high amounts of phenolic compounds; moreover, they showed that treated OOMW bare reduced phytotoxicity, although it contained more toxic phenols when compared to the untreated

OOMW. This means that the oxidation treatment with *Pleurotus spp* enhanced the overall chemical burden and putative toxicity potential, but such enhancement did not translate into actual phytotoxic potential (Tsioulpas et al. 2002). OOMW decolouration and detoxification were also achieved by *Pleurotus* and *Ganoderma* spp. due to their intense enzymatic activities. In this context, despite *Pleurotus* spp. took more time to produce laccase (which means a longer adaptation period when compared with other fungus genera), at the end it translated in better yields compared to *Ganoderma* spp. (Ntougias et al. 2012).

One aspect deserving detailed attention is the fact that, by applying fungi in environmental matrices, their metabolic action can be inhibited due to the high concentration of contaminants. In addition, there are concerns with the possible introduction of exotic species in the receptor media that could induce a local imbalance. This was the motto of the work by Duarte et al. (2014), who addressed this drawback by developing a silica-alginate-fungi biocomposite using *P. sajor caju* that prevents fungi release, protecting both the organism and the surrounding environmental matrix. Besides the capacity of this biocomposite to reduce COD, concentration of phenolic compounds, sterols and fatty acids, it can be reused in other treatments. However, the biocomposite was unable to remove high molecular weight compounds, which is a disadvantage in what regards these wastewaters.

Bacteria have also been considered for OOMW biotreatment approaches. For example, a study using *Ralstonia eutropha* towards the degradation of both phenolic compounds and COD in OOMW was conducted on the basis of previous evidences of the ability of these bacteria to grow in a 20% dilution of OOMW (Jalilnejad et al. 2011). The authors detailed that previous acclimation to phenol and aeration enabled the growth of the microorganism, promoting a better biodegradation performance as the inhibitory effect of OOMW was reduced. In another study, strains of *Ralstonia* sp. and *Pseudomonas* sp., both individually and combined in a co-culture, have also been analysed towards a sample of natural OOMW (which have been treated by reverse osmosis) and a sample of OOMW provided by an anaerobic treatment facility. The results showed that the bacteria were able to mutually complement each other in completely removing the monocyclic aromatic compounds from the first sample and some low-molecular-weight aromatic compounds found in the second sample, respectively (Di Gioia et al. 2001). *Paenibacillus jamilae* was tested towards the production of exopolysaccharides (microbial polymers with great biotechnological potential at the cellular level) in OOMW as growth medium (Aguilera et al. 2008). The results showed that *P. jamilae* both produced the polymer and detoxified OOMW. Furthermore, strains of *Raoultella terrigena* and *Pantoea agglomerans* were isolated, selected and used for the biotreatment of OOMW – the success of this approach was demonstrated as the remaining residue was in agreement with the EU legislation, the authors then arguing on its potential to irrigate agricultural lands as a fertilizer (Maza-Márquez et al. 2013).

An integrated approach based on aerobic and anaerobic treatments was proposed by González-González et al. (2015). First, the aerobic pre-treatment was performed by using already existing microorganisms in OOMW, thus profiting from adaptation. Then, fractions of this treated effluent were exposed to the anaerobic treatment. Polyphenolic removal reached 90% after inoculated OOMW aeration for 7 days and it was possible to recover approximately 30 % of water following anaerobic digestion. Another promising microbial-bacterial consortium was proposed as an OOMW biotreatment combining the bacteria *Raoultella terrigena* and *Pantoea agglomerans* with the microalgae *Chlorella vulgaris* and *Scenedesmus obliquus*. Considering that microalgae consume carbon dioxide and that bacteria needs oxygen to further biodegrade pollutants, the reduction of the phenolic concentration in OOMW was promoted under these conditions (Maza-Márquez et al. 2014). In summary, treatments with microbial consortia rather than single microorganisms seem to be more advantageous: first, a higher abundance and diversity of organisms normally renders the process more efficient since different organisms target a wider group of organic compounds; second, their enhanced stability and metabolic activity can improve the final outcome of the process (Sivasubramanian & Namasivayam 2015; Maza-Márquez et al. 2014).

Enzymatic treatments have also been suggested as an interesting solution to remediate OOMW provided the release of polyphenolic compounds and fermented carbohydrates, the latter susceptible to go under anaerobiosis and produce biogas. In addition, this treatment does not require chemical additives nor a controlled operating equipment to monitor basic parameters such as pH and temperature (Dammak et al. 2016).

Biosorption is a physicochemical process by which a solid surface of a biological matrix interacts with a sorbate resulting in the decrease of its concentration (Fomina & Gadd 2014). This process is a common approach used in the treatment of contaminants (i.e. the sorbates) including compounds such as metals and metalloids, dyes, pesticides, phenols, radioisotopes and pharmaceuticals (Fomina & Gadd 2014). Literature on biosorption to treat OOMW is scarcely available suggesting a low potential of this bioremediation process as compared with other methods described above. The few studies that exist are based in the use of bioreactors with microorganisms that are able to adsorb phenolic compounds which can then be valued by being reused in different industrial activities, namely in the pharmaceutical and cosmetic sector (Roig et al. 2006). For example, Chiavola et al. (2014) proposed a sequencing batch reactor based on active sludge. This bioreactor could achieve high performances, including complete removal of biodegradable organic contents of the tested OOMW, but residual polyphenol levels were still unsatisfactory even when the bioreactor was coupled with a membrane separation stage.

2.3.2.3. Integrated treatments for OOMW

Physicochemical treatments are more efficient towards discoloured OOMW, but their incapacity of detoxifying waters and wastewaters is an important shortcoming. To overcome it, integrated systems combining chemical and biological treatments have been investigated. There are already several studies covering this thematic, with huge diversity of combinations being suggested: photo-Fenton and fungi (Justino et al. 2009); electrochemical and aerobic treatment (Hanafi et al. 2011); ozonation and ultraviolet, ozonation and aerobic treatment and ozonation/ultraviolet followed by aerobic degradation (Lafi et al. 2009); anaerobic digestion with Fenton and electro-Fenton (El-Gohary et al. 2009; Khoufi et al. 2006). Overall, these studies conclude that an integrated approach enhances the removal of phenolic compounds, COD, and allows a reduction in the toxicity of the wastewater. Justino et al. (2009) suggest that the photo-Fenton process could be applied as a tertiary treatment in a wastewater treatment plant due to its large efficiency in decolorate OOMW, and also the lowest consumption of water. However, when photo-Fenton was applied before the fungi treatment, *P. sajor caju* was not capable of achieve the same COD, phenols and toxicity removal, and this was attributed by the authors to the increasing wastewater toxicity promoted by the Fenton process. Lafi et al. (Lafi et al. 2009) refer that ultraviolet/ozonation treatment is more effective than the ozonation followed by aerobic degradation in removing phenols. This experiment also recognizes ultraviolet/ozonation followed aerobic degradation as the best-known approach regarding COD, as they achieved a removal rate of 90.7%.

2.4. Conclusions

Our survey presents a detailed analysis regarding management and treatment processes that can be applied to OOMW. Taking into consideration the technological costs associated with some treatments, the lack of legislation, the lack of environmental education and the financial limitations of small producers, it is obvious that there is a long way to go regarding the olive oil industry and the management of its externalities. In fact, major reasons behind the lack of treatment implementation in olive oil mills are the existence of small-scale industries combined with the seasonality of the production process, mill’s geographical dispersion and the reduced economic power of their managers, who in most of the cases run a familiar business and cannot afford equipment investments (Ioannou-Ttofa et al. 2017). The fact that there is still lacking a commonly accepted cost-efficient treatment that fulfils environmental quality standards, renders the environmental hazardous potential of OOMW an ongoing problem especially in the Mediterranean region (Paraskeva & Diamadopoulos 2006).

Still, treated OOMW presents a great opportunity to be used in agricultural activities, thus reducing the necessity of water and consequently its costs to the olive mill (Ioannou-Ttofa et al. 2017). Furthermore, the possibility of recover phenolic compounds open doors to other scientific challenges, as these compounds can be part of a plethora of applications: food, cosmetics, biofuels and agrochemicals. The optimization and implementation of these ideas can lead the olive oil industry to reach zero waste and, ultimately, a sustainable solution for both economy and environment.

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CHAPTER 3. Bioremediation of olive oil mill wastewaters by the invasive bivalve *Corbicula fluminea*

Abstract

Bioremediation has been arising as a successful wastewater treatment in what concerns contamination events. Olive oil mill wastewaters (OOMW) are amongst the most concerning industrial wastewaters in the Mediterranean region mainly due to its seasonal intense production volumes, leading to numerous problems in both freshwater systems and soils. Bivalves, in particular, present one of the most attractive solutions to integrate remediation strategies due to their notable filtration capabilities and often their wide tolerance towards several chemical contaminants. The aim of this study is to explore the potential of *Corbicula fluminea*, a freshwater invasive bivalve, as a bioremediation agent towards OOMW. In this way, *C. fluminea* was exposed to this wastewater, and untreated and biotreated fractions were compared in terms of (i) their chemical composition; (ii) bioaccumulation in bivalve soft tissues and shells; and, finally, (iii) ecotoxicity, using standard organisms (the bacterium *Allivibrio fischeri*, the microalgae *Raphidocelis subcapitata*, the cladoceran *Daphnia magna* and the macrophyte *Lemna minor*). Results indicate that soft tissues and shells are proper recipients for the olive oil contaminants; also, the toxicity was generally reduced with the biotreatment. Overall, this study presents the first approach linking OMWW with *C. fluminea* as a potential bioremediation agent, with some promising results regarding the use of this bivalve in remediation strategies.

Keywords: *Corbicula fluminea*; centrifuge washing effluent; biofiltration; bioaccumulation; soft tissues; shells

3.1. Introduction

Bioremediation is the use of living organisms to remove environmental pollutants or, at least, to mitigate their impacts on the ecosystems. This strategy possesses numerous advantages, namely (i) it is often a simple and easy approach; (ii) it is a natural, thus an environmentally safer treatment, bearing low toxicity; (iii) and it is often cost-efficient as a long-term solution (Gifford et al. 2007). Indeed, the use of the freshwater bivalve *Corbicula fluminea* in bioremediation processes through its filtration and accumulation capacity was theoretically proposed several years ago (Doherty 1990).

In the context of the use of biofiltration as a bioremediation strategy, the filtration process is directly related to the ability of an organism to establish a dynamic equilibrium between its external and internal environments (bioaccumulation), that results from the regulation of the uptake, excretion, storage and degradation processes, as well as their respective interaction with each type of chemical compound (natural or synthetic) (Guo & Feng 2018). In fact, in the wild, filtration is a highly important ecosystem service – by clearing the medium through the filtration of suspended organic matter, filter-feeders clear and improve water quality while feeding (Way et al. 1990). Bivalves like the Asian clam *C. fluminea* are active filter-feeders, indeed well known for their high filtration capacities (Marescaux et al. 2016); in addition, they are recognised as good bioindicators of freshwater habitats for their role as representative sentinels of both sediments and water column (Bertrand et al. 2017), meaning that they withstand relevant contamination levels and can accumulate these contaminants significantly in their tissues and shells.

Different organic compounds and wastewaters have been used in bioremediation assays with *C. fluminea*. Polycyclic aromatic hydrocarbons are highly persistent and toxic contaminants, with potential of being bioaccumulated and biomagnified through the food chain (Muir & Howard 2006; Lohmann et al. 2007), that were already tested in this context. In the work of Silva et al. (2016), *C. fluminea* was exposed to ashes of post-fire runoffs with the intent of discussing the impacts of wildfires towards the Asian clam and the role of the clam in the recovery of affected ecosystems. *C. fluminea* successfully filtered PAHs, generally reducing their concentration in the water. Furthermore, *C. fluminea* has already proven to accumulate the herbicide atrazine in its tissues in a study focused in the clam's potential as biomonitors (Jacomini et al. 2006); and to remove triclorocarban (present in personal care products, soaps and detergents) and the pharmaceutical propranolol from water (Ismail et al. 2014). The capacity of the Asian clam to assist wastewater treatment has been also receiving some attention. Ferreira et al.

(2017) found that *C. fluminea* could reduce the COD content from a winery wastewater, after an initial chemical approach through Fenton process. Their results showed that the Fenton process could reduce the toxicity of the wastewater and that the clam reduced COD in both untreated and pre-treated wastewater, with better performances in the latter case. Winery wastewaters share several characteristics with OOMW due to their seasonal production and high contents of organic matter that undergo changes over time. Still regarding wastewaters, bivalves are also apparently a suitable approach to remove microorganisms (major components in these matrices) that may represent a threat to human health. *E. coli* levels in aquatic matrices are used as indicators of the presence of faecal bacteria, and their reduction was successfully achieved by *Anodonta californiensis* and *C. fluminea* (Ismail et al. 2015; Ismail et al. 2016; Gomes, Lopes, et al. 2018). Indeed, bacteria in general can represent an important food resource to the Asian clam, especially when phytoplankton availability is reduced (Vaughn & Hakenkamp 2001). As phytoplankton levels are high, *C. fluminea* seems as well to have potential as an agent for potentiating the recovery of eutrophic systems. For example, Cohen et al. (1984) found that *C. fluminea* was responsible for the removal of 30% of chlorophyll in only 2 hours in natural eutrophic waters. The use of bivalves has also been unleashing interest in the aquaculture field, where they are used to clear water tanks, protect fish farming cultures from adverse chemical and microbial impacts and, at last, prevent human health problems (Gifford et al. 2004; Bert 2007; Sapkota et al. 2008). Despite the effectiveness of bivalves in improving water quality in the context of aquaculture already begun to be addressed (Martínez-Córdova et al. 2011), studies using *C. fluminea* as a bioremediation agent for organic contaminants hasn't been widely explored.

The design of existing wastewater treatment plants did not consider the removal of micropollutants, instead their target are suspended solids, organic/faecal matter, grasses and nutrients. Meanwhile, the development of highly persistent and toxic chemicals that are largely discharged in the aquatic systems created a new problem for these facilities, since the available methods do not efficiently remove contaminants and/or they do not support and/or mitigate their toxicity (Binelli et al. 2015). In this context, bivalves' filtration capabilities and their high resistance to several environmental factors and contaminants make them a suitable low-cost solution for water and wastewater treatments. Moreover, the controlled integration of an alien species such as *C. fluminea* in industrial wastewater treatment facilities renders the opportunity of offsetting its typical fouling-derived damages (see e.g. Rosa et al. 2011), thus configuring a successful add-on to pest management programmes.

The purpose of the present study was to assess the filtration capacity of *C. fluminea* towards OOMW at a preliminary lab scale. To this end, a biotreatment experiment was carried out and the test solutions, as well as the organisms were assessed following chemical and biological approaches: (i) a broad chemical study to explore the main properties, structure and composition of the wastewater and the Asian clam (soft tissues and shells), while assessing the effectiveness of the biotreatment; and (ii) an ecotoxicological assessment using standard organisms to determine and compare their responses following exposure to untreated and biotreated OOMW. To the best of our knowledge, this is the first study focused on the bioremediation potential of *C. fluminea* against OOMW.

3.2. Materials and Methods

3.2.1. Wastewater Sampling

Wastewater samples were provided by a regional olive oil producers' association from the North of Portugal (AOTAD). This local mill provided a wastewater fraction from the washing of centrifuges, hereinafter designated as centrifuge washing effluent (CWE). Samples were collected after the olive oil extraction, then transported to the laboratory and stored at 20°C until being used in the experiment. Immediately prior to its use in the biotreatment test, all samples were filtered through a one-millimetre sieve.

3.2.2. Test Organisms

C. fluminea individuals were collected in Casal de São Tomé, Mira, Portugal (40°25'06.90''N, 8°44'13.18''W). Locally, clams were chosen according to their shell length (between 18 to 30 mm), since this is the size yielding more efficient filtration rates in this population (Castro et al. 2018). These individuals were transported to the laboratory along with a sample of local water. They were kept for a two-week quarantine period, gradually acclimating to the laboratory conditions: clams were firstly kept with equal proportions of dechlorinated municipal water and local field water, and then the later was progressively replaced by dechlorinated municipal water – twice a week the water was fully renewed. Also, the clams were kept with continuous aeration, temperature (20 ± 2°C) and photoperiod (16h light: 8h dark) and were fed *ad libitum* with *Raphidocelis subcapitata* suspensions twice a week.

Ecotoxicological assessment of the effluent (see the methods below in section 3.1.5) was performed through the exposure of *Allivibrio fischeri*, *Raphidocelis subcapitata*, *Daphnia magna* and *Lemna minor*, all recognised standard aquatic test species. *A. fischeri* was used after reconstitution of the lyophilised stock supplied within the Microtox® kit. The microalgae *R. subcapitata* has been continuously cultured in synthetic Woods Hole MBL medium (OECD 2006a; Stein 1973) renewed weekly, at 20 ± 2 ºC and under a 16h ^{L}: 8h ^{D} photoperiod. The cladoceran zooplankter *D. magna* has been maintained as a monoclonal culture in synthetic hard water medium (OECD 2004; ASTM 1980) added standard algae extract and vitamins (Loureiro et al. 2011). These cultures were renewed and fed (with *R. subcapitata* suspensions at a 3 x 10^4 cells/ml ration) three times per week and were kept under constant temperature (20 ± 2°C) and photoperiod (16h ^{L}: 8h ^{D}) conditions. The macrophyte *L. minor* was cultured in synthetic Steinberg medium (OECD 2006b) renewed weekly, at 20 ± 2ºC and photoperiod (16h ^{L}: 8h ^{D}) conditions.

3.2.3. Biotreatment Experiment

Prior to the biotreatment experiment, a preliminary mortality test was performed to establish the highest effluent dilution eliciting no mortality. This test exposed 210 clams (ten clams per treatment, with three replicates each) to a range of concentrations (0; 1.56, 3.125; 6.25; 12.5; 25 and 50% v/v) of the test suspension diluted in dechlorinated municipal water (CWE) during 96 h. Baseline parameters like temperature, pH, conductivity and dissolved oxygen concentration were daily monitored, and clam's mortality was also assessed by verifying active siphoning activity and resistance to valve opening forced by a blunt dissection needle. This preliminary test showed that clams withstand for 96 h in 50% CWE (13.3 ± 8.8% mortality), which shows a good tolerance capacity. Considering this result, along with the intent of maximise the biotreatment capacity of the clams towards a more concentrated test solution, the final biotreatment experiment considered a seven days' exposure period to 80% CWE; with sampling occurring in the beginning (day 0), middle (72h h, day 3) and end (day 7) of the experiment.

Clams were not fed during the experiment so that filtration rates could be maximized and dedicated to the organic load in CWE. The vessels were kept under continuous aeration, constant temperature (20 ± 2°C) and photoperiod (16h ^{L}: 8h ^{D}). Each treatment vessel contained 20 clams in 1.5 L of test suspension, and three replicates were established per treatment: CWE and CTR - Control composed of blank dechlorinated municipal water. Every day, clam mortality was assessed as described above. Baseline parameters (pH, temperature, conductivity and dissolved oxygen concentration) were measured at the beginning, middle (day 3) and end of the experiment with a multiparameter probe (Aquaprobe® AP-2000). Samples of the test solution were also collected following this timeline and preserved at -20°C to further chemical analysis (see section 3.3.4) and ecotoxicological testing (section 3.3.5). In addition, *C. fluminea* organisms were collected at the beginning (sub-sample of the batch that was used to perform the experiment) and at the end of the experiment. Soft tissues and shells were separately preserved at -80°C and -20°C, respectively, for organic chemical quantification (section 3.3.4).

3.2.4. Physicochemical Analyses

Colorimetric methods as the absorbance at 270 nm, 465 nm and 320 nm for quantifying aromatic compounds, coloured compounds and coloured dissolved organic carbon (CDOC), respectively, of CWE at the beginning, middle (day 3) and end of the biotreatment (Shimadzu UV-1800, UV Spectrophotometer). Chemical oxygen demand (COD) concentrations were also measured for the same sampling time points (Hanna Instruments 93754B – high range: 0-15000 mg/L; multiparameter bench photometer C214), following the USEPA method 410.4 (USEPA 1993). Ammonium concentration was measured at the end of the test (PC Multi Aqualytic®) to monitor its natural production, either through excretory by-products or by the decomposition of organic matter, including dead clams.

Fourier-transform infrared spectroscopy with attenuated total reflectance (ATR-FTIR) was performed in CWE and control samples taken at the beginning and end of the experiment, using a Perkin Elmer (USA) Spectrum BX FTIR instrument; a resolution of 4 cm^{-1} within the 4000 - 500 cm^{-1} range was established, and water was used for the background spectrum. Soft tissues of *C. fluminea* were previously cold dried and lyophilised during 72 hours at -85°C and 0.080 mbar (Telstar Lyo Quest). Then, ATR-FTIR spectra were recorded between 3850 and 600 cm^{-1}, with a resolution of 4 cm^{-1}. In addition, ATR-FTIR was performed in the shell matrix of the Asian clam, with a resolution of 4 cm^{-1} within the 4000 - 500 cm^{-1} range. In both soft tissues and shells, air was used for the background spectrum.

Scanning electron microscopy incorporated with an energy-dispersive X-ray spectrometer (SEM-EDX) and X-ray diffraction (XRD) analysis were performed in shell samples of the Asian clam (taken at the beginning and the end of the experiment) that were previously crushed with a grinder until powdered. SEM (Hitachi S4100, SU-70 microscope) operating at 8.0 kV and an EDX detector (Bruker, QUANTAX 400) were used to compare the elemental composition between organism shells from the CTR and CWE treatments. XRD (Philips X'Pert MPD/MRD) was performed using a powdered diffractometer operating at 40 mA and 45 kV, with Cu Kα radiation filtered by Ni. XRD patterns were recorded with a step size (2ϴ) of 0.0260 and a time per step of 56.8650 seconds. XRD was used to study the crystalline structure of the shell of the Asian clam in terms of its chemical signature and crystallography.

3.2.5. Ecotoxicological Assessment

Pre- and biotreated CWE samples were taken at the beginning and the end of the biofiltration experiment, and then tested for their ecotoxicity using standard tests.

The *A. fischeri* luminescence inhibition test was performed using the commercial Microtox® test kit, following the liquid-phase 81.9 basic test protocol (AE 1998). Bacteria were exposed to the samples, and luminescence inhibition was assessed following 5, 15 and 30 minutes of exposure.

Growth inhibition of *R. subcapitata* was assessed following the OECD guideline 201 (OECD 2006a) adapted to 24-well microplate use (Geis et al. 2000). The microplates were incubated for 96 h at 23 ± 1 °C, under continuous illumination. The microalgae were exposed to CWE samples, comprising a range of dilutions in dechlorinated municipal water of 12.5, 25, 50 to 100%. A blank control of Woods Hole MBL medium was also prepared, and nutrient supply was provided in all wells to ensure the required nutrient levels in tested samples as in the control. All treatments were tested in triplicate, and each replicate was set with 990 µL of test solution and 10 µL of concentrated inoculum (10^6 cells/mL to start the test with a standard cell density of 10^4 cells/mL). Throughout the experiment, microplates were mixed twice a day to re-suspend deposited cells. At the end of the experiment, the algal density in the wells was estimated through microscopic cell counts.

Immobilisation of *D. magna* was assessed following the OECD guideline 202 (OECD 2004). Daphnids were exposed to CWE samples, within a range of dilutions in distilled water of 12.5, 25, 50 to 100%. Exposure of *D. magna* was run for 48 h at 20 ± 2°C. The assay consisted of a blank control with distilled water supplemented with the appropriate nutrients as indicated for ASTM, and the four CWE treatments (also supplemented with nutrients), each with four replicates containing five animals (20 neonates per treatment) less than 24-h old and born within 3^{rd}-5^{rd} brood in laboratory cultures. At the end of the experiment, the immobilised daphnids were counted.

Growth inhibition of *L. minor* was assessed following the OECD guideline 221 (OECD 2006b) adapted to 6-well plates use (Kaza et al. 2007). *L. minor* were exposed to pre- and biotreated CWE samples, within a range of dilutions comprising 12.5, 25, 50 to 100%. Exposure of the macrophyte was kept during one week at 23 ± 1°C, under continuous illumination. A blank control was performed with Steinberg medium and the corresponding nutrient spike was added to all CWE treatments. All treatments were run in triplicate, each replicate with nine fronds at the beginning of the assay. At the end of the assay, fronds in each well were counted and their dry weight was determined for growth calculations.

3.2.6. Data Analysis

Variation in the organic compounds of CWE were analysed through paired t-tests between: (i) biotreated (day 7) and untreated (day 0) CTR samples; and (ii) biotreated (day 7) and untreated (day 0) CWE samples, with the aim of addressing the significance of the biotreatment experiment in terms of the molecular composition of the test solution (CTR or CWE). These tests were independently run considering the peak areas calculated for each region of the ATR-FTIR spectra.

A similar approach was followed to assess the putative differences in the molecular composition of soft tissues or shells at the beginning and the end of the experiment: paired t-tests confronting the peak areas within each FTIT-ATR region at the end of the experiment in the CTR and CWE with the corresponding areas recorded at the beginning of the experiment. The variation of molecular composition of soft tissues and shells through the experiment was determined as the difference between the final composition (day 7) of soft tissues or shells and their initial composition (day 0), respectively. This allowed a clearer graphical interpretation of the differential patterns between CTR and CWE, both for tissues and shells, as well as a direct assessment of statistical differences between these two treatments simple t-tests.

All analyses were performed using the Microsoft Office 365 Excel. A significance level of 0.05 was considered in all statistical analyses.

3.3. Results and Discussion

3.3.1. Physicochemical characterisation of the wastewater through the biotreatment experiment

In the biotreatment experiment, at day 3 (72 h), there was no record of any dead clam for both CTR and CWE treatments; however, at the end of the assay (7 days), a 60.0 ± 8.7% mortality was observed for 80% CWE. This was an expected result based on the water quality parameters followed throughout the biotreatment (Figure 3.1). Temperature was constant throughout the experiment in all treatment samples; pH was constant in the control through the experiment (pH values between 7.16 and 7.64), while it was slightly acidic in CWE at the beginning of the experiment (pH of 4.29 ± 0.022) with an increase being noticed through the experiment reaching 6.52 ± 0.11 at the end (Figure 3.1). pH levels registered for untreated CWE (day 0) were within the expected range for these wastewaters (Justino et al. 2012). Conductivity slightly increased in the control (between 99.33 ± 1.25 µS/cm at day 0, and 189.33 ± 10.21 µS/cm at day 7); in the case of CWE, conductivity was higher compared to the control, increasing littler from day 0 to day 7 (from 1016 ± 44 µS/cm at day 0, to 1444 ± 48 µS/cm at day 7) due to the condensation of salts. Dissolved oxygen levels also increased from the beginning to the end of the test in CWE, which certainly reflects the setting of aeration; while in the control they remained similar and constant. Absorbance at 270 nm (characteristic of aromatic compounds) in CWE increased for the first 72 h, returning to initial values at the end of the experiment, though these levels were always higher than those noticed in the control, where maximum absorbance was noticed at the end of the experiment, possibly as a result of clam's metabolism and excretion (Table S1). Absorbance at 465 nm (characteristic of coloured compounds) was generally constant throughout the assay in CWE and in the control, with control levels being relevantly lower than those found in CWE (Table S1). Also, CWE absorbances at 465 and 270 nm are within the same range, indicating that the fraction of aromatic compounds is probably associated with the coloured characteristics of CWE. In the case of CDOC (320 nm), both CTR and CWE experience an increase in their levels in day 3, followed by a reduction at day 7 (Table S1). These alterations indicate that after an increase in the organic burden of CWE at day 3, there was a reduction at the end of the biotreatment, probably associated with the biofiltration role of the Asian clam.

The higher N levels in CWE at the end of the experiment (0.64 mg/L of N) were likely caused by a contribution of the decomposition of dead clams during the last four days of the treatment. In fact, the mortality of clams is known to enhance the levels of nitrogen, as already reported in some studies (Guo & Feng 2018; Pipolo et al. 2017).

However, the final level of N in this study is residual, complying with the legal limit for OOMW discharges - 15 mg/L of N (total Kjeldhal nitrogen, TKN) according to the National regulation (Law-by-Decree no. 236/98 of August 1, 1998).

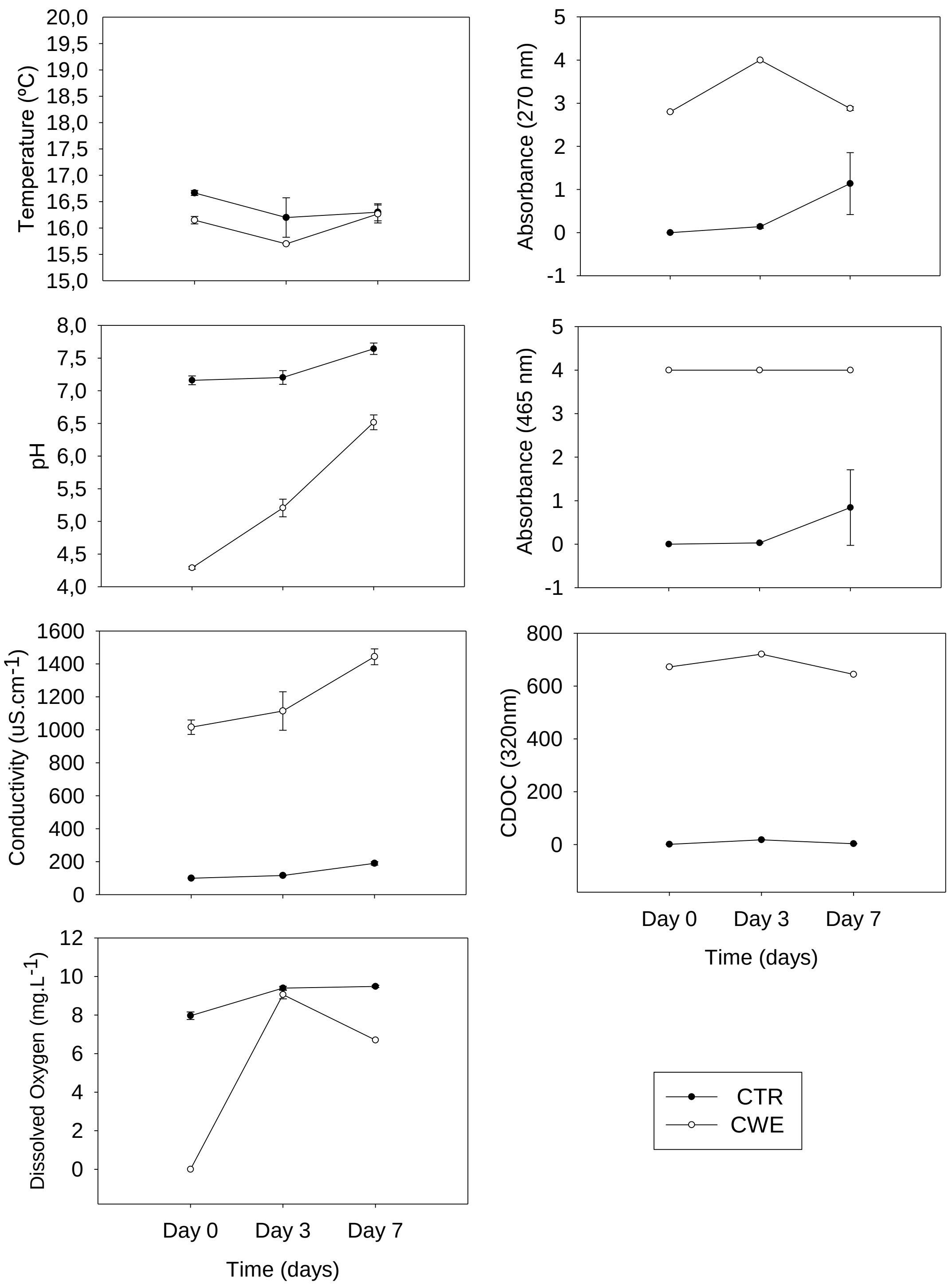

Figure 3.1. Water quality parameters recorded at the beginning (day 0), middle (day 3) and end (day 7) of the biotreatment experiment, in the control (CTR) and in the OOMW treatment (centrifuge washing effluent, CWE). Each point represents the average of three experimental replicates and the error bars represent the corresponding standard deviation.

Chemical oxygen demand (COD) was analysed in 80% CWE samples. COD levels reached its maximum in the middle of the biotreatment experiment (72 h, day 3; COD = 250 g/L) compared to 180 and 20 g/L at the beginning and the end of the experiment, respectively. In fact, oxidative reactions may generate some recalcitrant compounds, which could explain this augmentation (Ferreira et al. 2017) through the third day of exposure but, since there was no clam mortality at this point, these COD level changes should rather be attributed to the microbial communities present in CWE. CWE possesses a great amount of sugars in its composition, which could stimulate the microbial activity and lead to the decomposition of simple molecules (McNamara et al. 2008), resulting in higher content of complex/non-degradable organic compounds and, thus, higher COD levels. Then, at the end of the biotreatment (day 7), COD levels were reduced to a minimum value which is one order of magnitude lower than as recorded before, while clam mortality reached 60.0 ± 8.7% at this time. Baseline parameters – temperature, pH, conductivity and dissolved oxygen – remained suitable for the survival of the clams at the end the experiment (day 7), according to the values reported by Mackie & Claudi (2010). Thus, this mortality event should be related to the inherent toxicity of CWE. The ingestion by the clams of toxic, complex organic compounds of CWE, such as phenolics explains both their mortality and the reduction of the COD/CDOC (lower levels of non-degradable compounds); this later record can additionally be promoted by concentration and sedimentation via pseudofaeces production by living clams.

Overall, the biotreatment successfully reduced 88,9% of the COD content of the untreated CWE corroborating previous evidences on the Asian clam capacity in reducing the contents of COD of highly recalcitrant organic effluents, like winery wastewaters (Pipolo et al. 2017; Ferreira et al. 2017). However, this reduction was not sufficient to fully meet the legal requirements for wastewaters discharges in natural aquatic systems: 150 mg/L O_2 according to the National regulation (Law-by-Decree no. 236/98 of August 1, 1998).

ATR-FTIR spectra of CWE and of the control (CTR) obtained through the biofiltration experiment are presented in Figure S1, and corresponding calculated areas are presented in Figure 3.2. In general, there was a reduction in all areas of the peaks corresponding to CWE from the beginning (day 0) to the end of the biotreatment (day 7), in all wavenumber regions. Moreover, these variations in CWE were statistically significant for all regions (paired t-test; $p < 0.05$); except in the region of 1180-900 cm^{-1}, where the change in areas was not significant (Table S2).

Regarding the CTR, there was an increase in the areas of the peaks from day 0 to day 4, and then a reduction from day 4 to day 7 in all wavenumber regions. Considering that the clams were not fed during the biotreatment, their metabolic rates should be minimal during the experiment which justifies the fact that the variations in the CTR were minor and not statistically significant (Table S2).

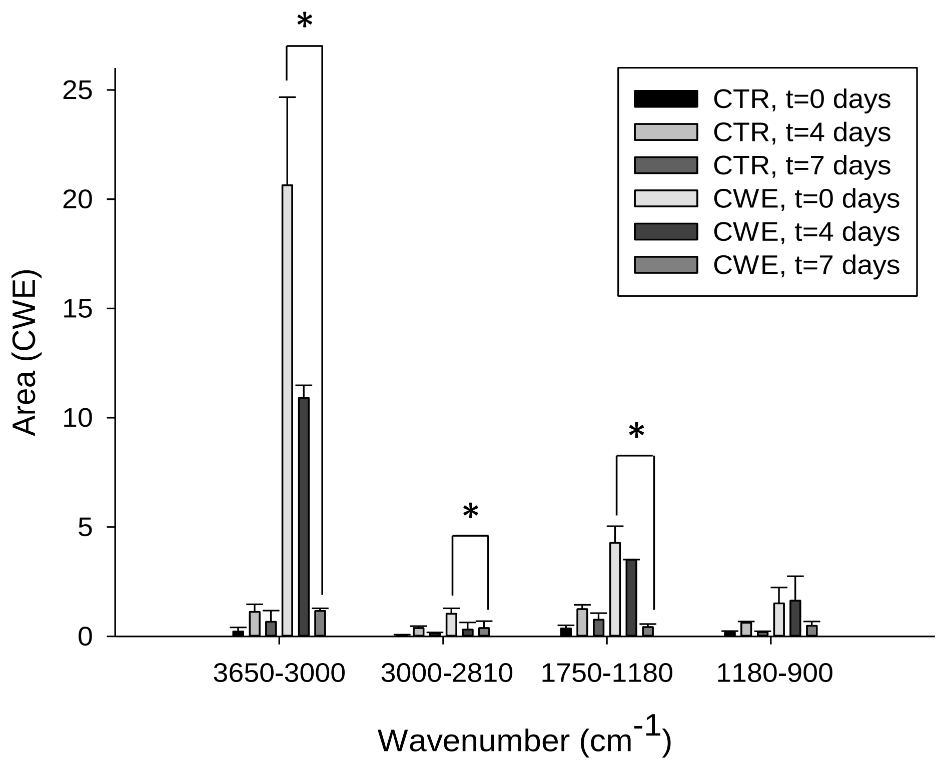

Figure 3.2. Mean variation in areas for the different regions of the ATR-FTIR spectra regarding CTR and CWE treatments through a 7-day filtration period with *C. fluminea*. Error bars represent the standard deviation. The asterisk denotes a significant variation occurring in the molecular composition of CWE through the experiment (paired-sample t-test comparing T0 with T7; $p < 0.05$).

The 3650-3000 cm^{-1} region is characteristic of OH stretching vibrational bonds (carboxyl, hydroxyl and phenols) and NH stretching vibrations in amides (Baddi et al. 2004; Hafidi et al. 2005; Droussi et al. 2009). The presence of higher levels of compounds with these features compared to other organic groups is expected in CWE according to its general chemical composition (Justino et al. 2012; Justino et al. 2010; Obied et al. 2005); also, phenolic compounds are the main toxic components present in these wastewaters (Justino et al. 2012). The significant decrease noticed in CWE regarding this ATR-FTIR region hence supports the potential of the clams to remove noxious compounds. The

3000-2810 cm^{-1} region is characteristic of aliphatic compounds (lipids, waxes and long chain structures). Within this region, there was observable significant decrease by more than half of the initial levels in fatty acid content of CWE through the biofiltration period. This can be related to the degradation of highly polymerized molecules into lower molecular weight structures induced by microbial communities that are surely present in the aqueous matrix (Hafidi et al. 2005; Venieri et al. 2010; Ntougias et al. 2013), which is likely to facilitate the biological uptake of these compounds by *C. fluminea*. Also, it is possible that the clam has filtered a fraction of fatty acids from CWE before using its own energy reserves to cope with the absence of feeding during the biotreatment. Vibrational stretching bonds characteristic of C=O, C=C and C=N (ketones, aldehydes, aromatics and amides) are within the 1750-1180 cm^{-1} region, and -C-O and CH_2 stretching (alcohols and polysaccharides) are associated with the 1180-900 cm^{-1} region. In both cases, CWE peak areas decreased through the experiment, significantly for the region within 1750 and 1180 cm^{-1}, suggesting a role of the clams in the removal of corresponding compounds from CWE (note that the peak areas in the CTR were lower and did not change significantly through the experiment). It is noteworthy that ATR-FTIR results from CWE are consistent with those obtained in COD: peak areas decreased in all wavenumber regions from day 0 to day 7, and so did the levels of COD in the same period of time, which further supports the role of the clam in the remediation of CWE.

3.3.2. Chemical characterisation of *C. fluminea* through the biotreatment experiment

3.3.2.1. Soft tissues of *C. fluminea*

Figure 3.3 shows the variation in the molecular composition of soft tissues of the Asian clam as pictured by ATR-FTIR analysis regarding exposures to the CTR and CWE. In practice, positive bars denote gains in soft tissues and negative bars denote losses at the end of the experiment compared to the records obtained at the beginning of the experiment with unexposed clams. From day 0 to day 7, the variation of chemical compounds in the soft tissues of both treatments did not differed significantly in any wavenumber region (Table S3). Moreover, differences between the molecular composition of soft tissues exposed to CTR (day 7) and CWE (day 7) were also not statistically significant, except for the region within 1750-1180 cm^{-1} (Table S4). The lack of significance of differences between CTR and CWE treatments for the region within 1180-900 cm^{-1} was particularly unexpected given the inverse pattern noticed by the variation data (Figure 3.3), but the low number of replicates used in this study and the large variation found between replicates in ATR-FTIR readings may concur to explain this apparent inconsistency.

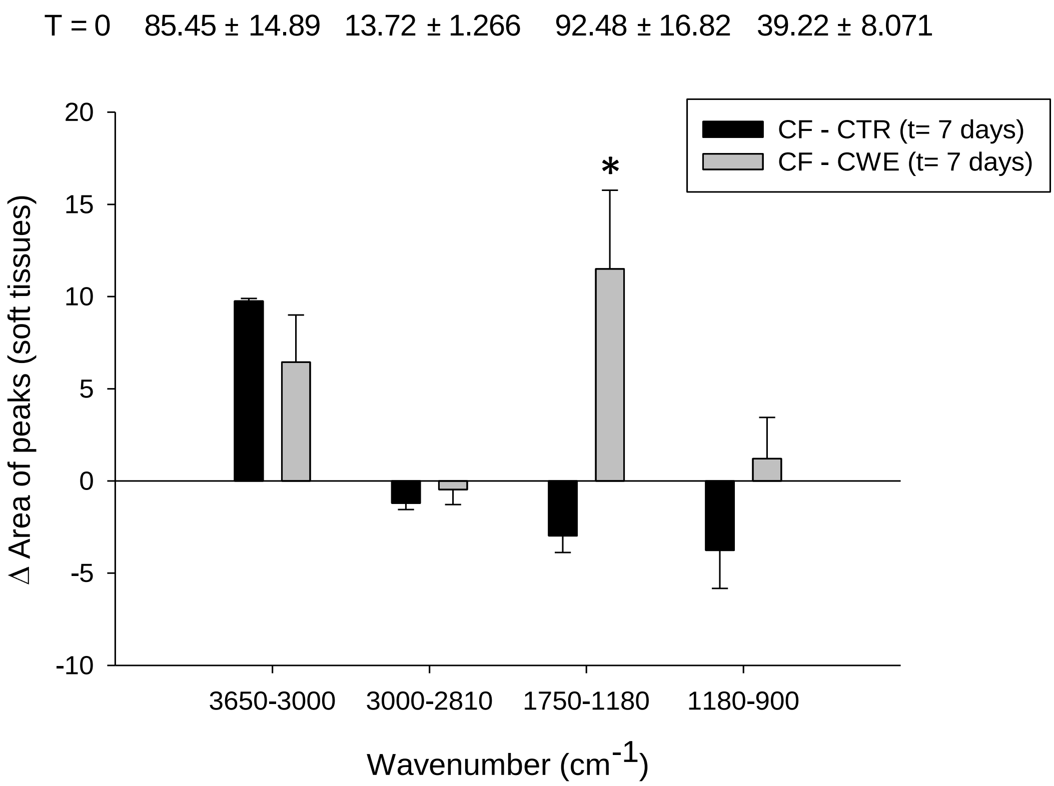

Figure 3.3. Mean variation in areas for the different regions of the ATR-FTIR spectra regarding soft tissues of *C. fluminea* following a 7-day filtration period over the CTR and the CWE in the biotreatment experiment. Readings at the beginning of the experiment served as the baseline to calculate the variation in peak area for each region, i.e. each record (n = 3 per treatment) obtained at the end of the experiment was subtracted the corresponding average record obtained for samples taken at the beginning of the experiment. Error bars represent the standard deviation. Asterisks denote significant differences between soft tissues exposed to CTR and CWE regarding the reading of areas at the end of the experiment (simple t-test; $p < 0.05$). In the top of the plot, the average areas calculated for each region at the beginning of the experiment in soft tissues are given for reference purposes.

The direct comparison of data reporting on losses from the water column (Figure 3.2) and gains in clam's soft tissues (Figure 3.3) allows a primary analysis on the capacity of the organism to internalise, metabolise and accumulate organic compounds of CWE. The transference of compounds from an aqueous matrix into the soft tissues can be achieved through a process known as bioconcentration, widely known in molluscs (Livingstone 1998). In addition, the close contact of the soft tissues of *C. fluminea* to a toxicant-loaded matrix such as CWE can also trigger biotransformation processes. Biotransformation is characterized as a series of enzymatic reactions that intend to cope with organic xenobiotics by converting their lipophilic nature in hydrophilic metabolites that are available to be excreted by the organism (Livingstone 1998) – in the case of clams, the elimination of contaminants is mainly performed by the kidneys (Gosling 2003).

The losses observed in CWE regarding OH and NH type of compounds (3650-3000 cm^{-1}; Figure 3.2) are hardly unrelated to the gains observed in soft tissue reflected in Figure 3.3. for the same region of the spectra, which is likely related with metabolic transformation of filtered compounds by the clams and their conversion into molecules of another organic nature.

The region of 3000-2810 cm^{-1} is associated with the C-H stretching vibrational bonds of aliphatic structures (lipids, waxes and long chain structures) (Baddi et al. 2004). There is no evidence of a relevant transfer of these compounds in the tissues of the clam for both CTR and CWE (Figure 3.3), which generally agrees with the very little content and variation found through the experiment in the test media (Figure 3.2).

The presence of ketones, aldehydes, aromatics and amides (present in proteins, for instance) is characterized by stretching vibrations in the frequency range of 1750-1180 cm^{-1}. According to Figure 3.3, allocation of these types of compounds to the tissues of the clam exposed to CWE is noticeable, which is consistent with the losses occurring from CWE (Figure 3.2) and significantly opposed to the loss observed from soft tissues in the CTR. The losses observed for the CTR could be explained by the absence of feeding during the experiment, which led them to depend on the mobilisation of energy reserves (in this case, probably deriving from protein mobilisation) to survive under starvation. In the meantime, the gains observed in CWE could be also associated to an increase of the metabolic stress induced by the exposure of the clams to this toxicant-loaded wastewater, triggering the production of enzymes to cope with this disturbance/pressure.

Lastly, the peaks within 1180-900 cm^{-1} are related with -C-O stretching and CH_2 bending, but they are also associated with the phosphate groups of nucleic acids present in the tissues of the clam. The increase of these compounds noticed in soft tissues exposed to CWE is consistent with the corresponding loss noticed from the exposure medium (Figure 3.2), but the same consistency cannot be recognised regarding the CTR, thus preventing a robust interpretation on the putative transfer from the media into clam soft tissues.

3.3.2.2. Shell matrix of *C. fluminea*

The areas from ATR-FTIR spectra corresponding to the shell compartment are shown in Figure 3.4. In general, the variation of the molecular composition of the shells exposed to

CTR (day 0) and CWE (day 7) was not significant, except for the CTR in the 1750-1180 cm^{-1} region (Table S5). Moreover, the differences in the molecular composition of both CTR and CWE treatments (day 7) were also not statistically significant (Table S6). Once again, the variation in readings is high and the sample size is low, which may have constrained this statistical outcome while a graphical analysis of Figure 3.4 seems to depict a different picture.

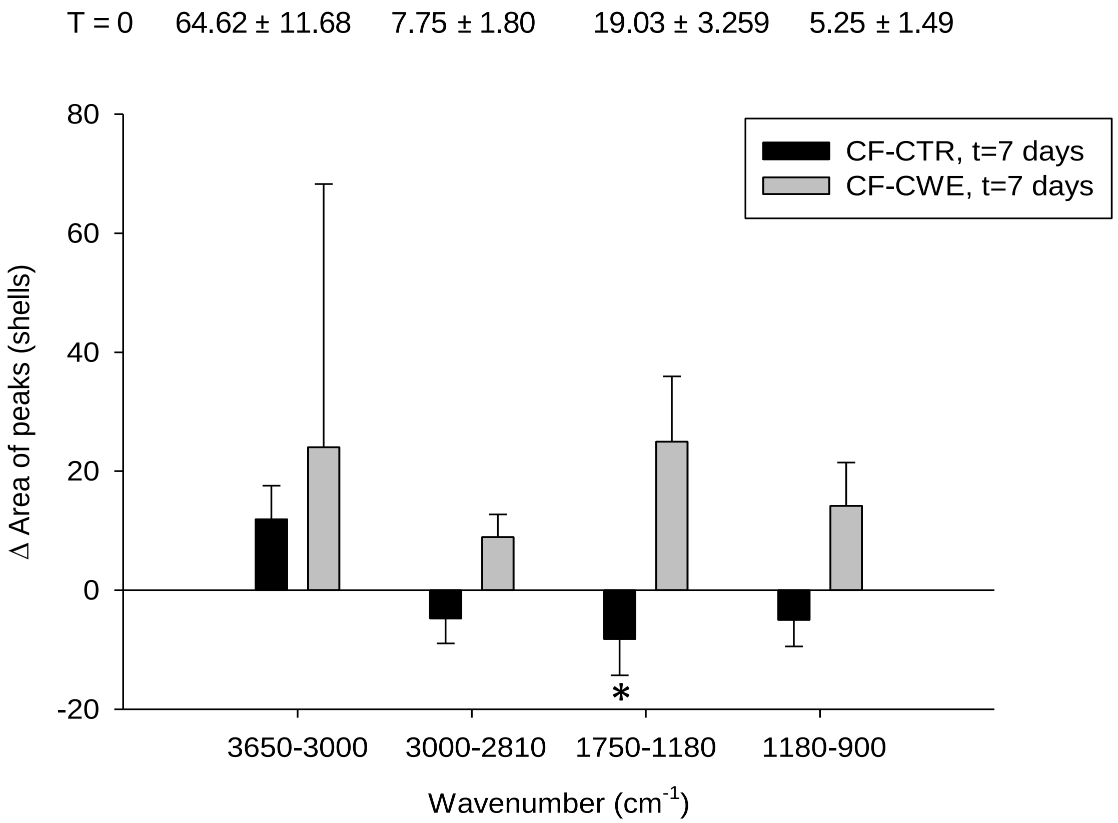

Figure 3.4. Mean variation in areas for the different regions of the ATR-FTIR spectra regarding the shells of *C. fluminea* following a 7-day filtration period over the CTR and the CWE in the biotreatment experiment. Readings at the beginning of the experiment served as the baseline to calculate the variation in peak area for each region, i.e. each record (n = 3 per treatment) obtained at the end of the experiment was subtracted the corresponding average record obtained for samples taken at the beginning of the experiment. Error bars represent the standard deviation. Asterisks denote significant variation through the experiment in shells exposed to CTR (paired t-test; $p < 0.05$). In the top of the plot, the average areas calculated for each region at the beginning of the experiment in shells are given for reference purposes.

In the 3650-3000 cm^{-1} region, both the CTR and CWE treatments show an increase that could be associated with the presence of carboxyl, hydroxyl and phenol groups (O-H vibrational bonds), characteristic of this range, through the experiment. Though the average area in CWE seems higher than that found in the control, the variation is very high, which prevents feasible distinction between these two treatments.

However, it is important to highlight that this region was the one also with the largest increase through the experiment for soft tissues, which suggests that changes in characteristic compounds occur in parallel or interactively between soft body and shells, i.e. that accumulation in shells depends on the metabolism of the living organism. The transference of compounds from soft tissues into the shell is a mechanism already verified in some studies with oysters exposed to metals (Huanxin et al. 2000). In fact, clams morphology enables a connexion between the shell and the tissues through the mantle margins, which means that a transference of pollutants to some level is likely to occur (Gosling 2003). In case of the CTR treatment, the calculated area is possibly related with the allocation of some metabolic by-products from the tissues.

The area within the 3000-2810 cm^{-1} region could indicate that a residual accumulation of lipids in the shells exposed to CWE, and lipids were already noticed as common in the composition of mollusc shells (Suzuki & Nagasawa 2013). The positive variation in areas for the other regions of the CWE spectra (1750-1180 cm^{-1} and 1180-900 cm^{-1}), could be related with the allocation of proteins and polysaccharides, respectively. It is noteworthy that for all these three latter regions, control shells lost compounds while there was a gain in CWE shell (Figure 3.4) in parallel to corresponding losses from the water (Figure 3.2). This is consistent evidence suggesting that the Asian clam shells have a significant role in accumulating filtered contaminants or the products resulting from their metabolism.

Yet, it is important to discuss the fact that the total losses observed in CWE (Figure 3.2) were not completely allocated to both biological compartments of the Asian clam (shells and soft tissues, Figures 3.3 and 3.4), which means that the remaining compounds are probably being concentrated and then released in the form of pseudofaeces to the outer medium. This bypass mechanism occurs since the clam does not ingest all particles incoming in the inhalant current. Depending on morphology or even on the chemistry of the filtered particles, the particles may not be properly processed by the gill apparatus and/or are rejected by the sensorial apparatus of the mouth, but rather involved in mucous produced by specialised cells of the demibranchs and directed to the exhalant siphon without ingestion (Way et al. 1990; Winter 1978; Riisgård 2001). From the clam’s perspective, this can be an important strategy to avoid internal exposure to noxious chemicals, and such a biological process may support in some cases the lack of correspondence between the losses found in CWE and the gains in soft tissues plus shells. In this dissertation, pseudofaeces were not directly approached due to the organic complexity that CWE poses in terms of its huge content of suspended solids and dark colour (Dermeche et al. 2013), which would have limited our capacity of clearly distinguish organic particles from pseudofaeces. Besides, the analysis of the molecular composition attributed to pseudofaeces also would not give us a direct correlation with our results from CWE, shells and especially soft tissues because a fraction of the organic compounds were indeed metabolized by the clam and transformed into other molecules, rather than being directly accumulated in their original form; also, the substances added by the mucous (mostly polysaccharides) would further confound the outcome.

X-ray diffraction results on shells exposed to the CTR treatment (Figure 3.5) informed that *C. fluminea* shells are 100% constituted by aragonite, which is a known natural polymorph of calcium carbonate, $CaCO_3$ (Spann et al. 2010). In fact, this result agrees with studies focused on the structure and composition of the shell of the Asian clam (Spann et al. 2010; Frenzel & Harper 2011). Furthermore, when comparing the results between CTR and CWE treatments, it is not possible to elucidate if the shell was in fact capable of adsorb CWE, as there are no evidences of an amorphous region between 20-30° in the structure of shells.

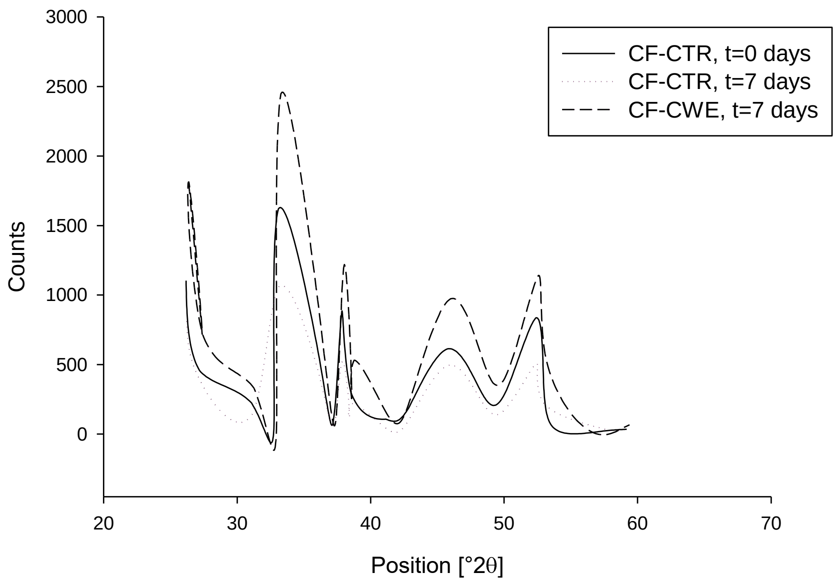

Figure 3.5. X-ray powder diffraction (XRD) spectra of the shells of *C. fluminea* before and after exposition to both CTR and CWE treatments.

SEM-EDX analyses regarding untreated/biotreated CTR and CWE samples are shown in Figure 3.6. All results are in agreement with its structural characteristics previously discussed (Rodriguez-Navarro et al. 2012; Kennedy et al. 1969; Li et al. 2017). Shells collected from CTR at the beginning of the experiment (day 0, Figure 3.6A) do not present noticeable differences after the biotreatment (Figure 3.6B); and the same is evidenced in the shells exposed to CWE at day 7 (Figure 3.6C) compared to day 0 (Figure 3.6A). Through the analyses of EDX results, it is visible the presence of calcium in the shells from all treatments, which agrees with XRD results (Figure 3.5) and with literature studies (Singh et al. 2016). The peaks of carbon and oxygen present in all graphics are most likely related with scattered radiation peaks – undesired radiation that normally occurs in organic samples due to their lower coefficients absorption x-rays. Relatively to the CWE treatment, it was expected that the shells at day 7 (Figure 3.6C) revealed other elements than calcium to further analyse the presence of adsorbed compounds. These results did not elucidate further on the capacity of the shells of the Asian clam to assist the remediation of CWE, as ATR-FTIR results (Fig 3.4) clearly indicate that shells are indeed capable of accumulating organic compounds.

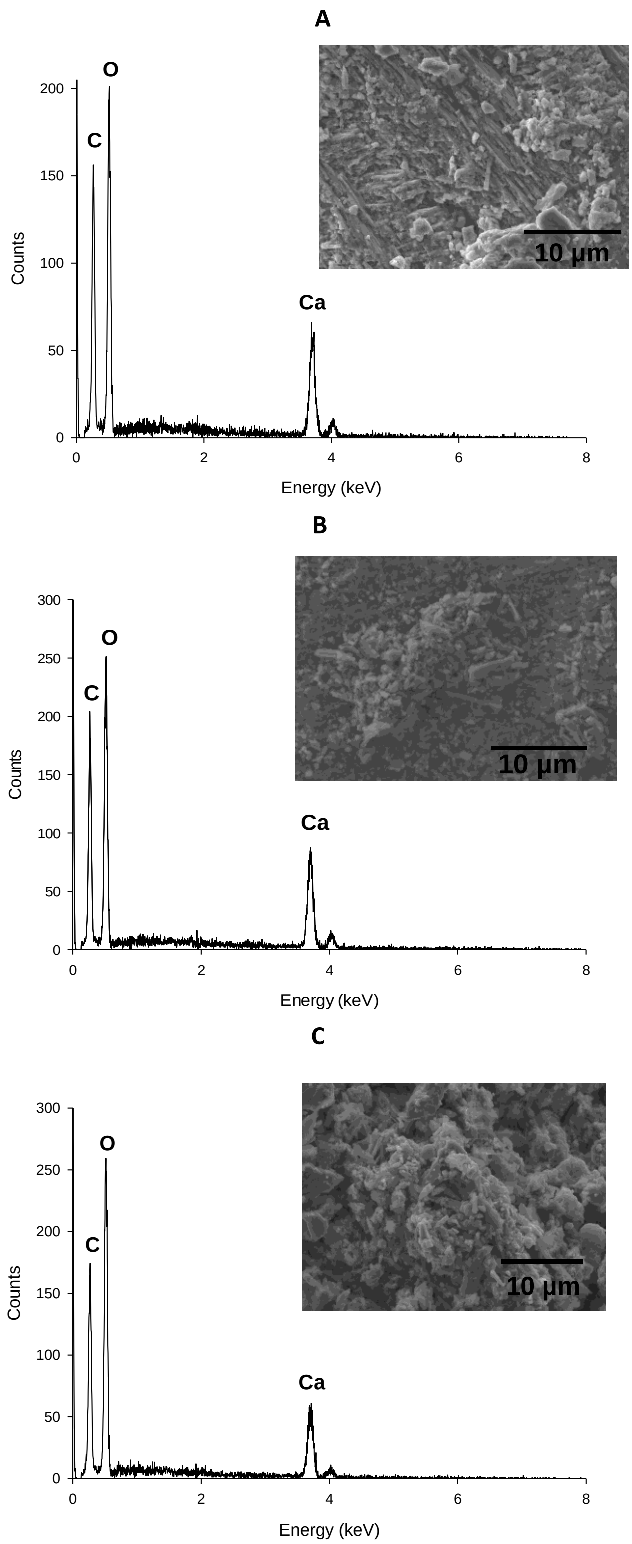

Figure 3.6. Energy dispersive X-ray (EDX) spectra with its corresponding images of scanning electron microscopy (SEM) performed in the shell of *C. fluminea*, before and after the biotreatment experiment: A) CF-CTR, t=0 days; B) CF-CTR, t=7 days; C) CF-CWE, t=7 days.

3.3.3. Ecotoxicological assessment of the wastewater

To complement the chemical analyses already presented, CWE was also submitted to an ecotoxicological assessment with standard organisms (Table 1): a bacterium, *A. fischeri*; two primary producers, the microalgae *R. subcapitata* and the macrophyte *L. minor*; and a primary consumer, *D. magna*. Results show that the bioluminescence of *A. fischeri* was nearly fully impaired later in the effluent concentration range for biotreated CWE compared to untreated CWE, this suggesting that the toxicity of CWE was lowered by the clams.

Table 1 – Maximum responses (Max. effect; %) along with the CWE or CTR (samples collected before and after the 7-day biotreatment with the clams) concentration (%) eliciting these responses as observed in bioassays with *A. fischeri, R. subcapitata, L. minor and D. magna.* Median effect concentrations (EC50) and corresponding confidence intervals (95% CI) are provided whenever the test results allowed their estimation via non-linear regression, using the least-squares method to fit the data to a general logistic equation.

		Untreated medium (t = 0 d)	**Biotreated medium (t = 7 d)**
***A. fischeri* luminescence inhibition (30 mins)**			
CWE	Max. effect (%)	>99 at 2.56%	>99 at 40.95%
	EC_{50}(%) (95% CI)	ND	ND
***R. subcapitata* growth inhibition**			
CWE	Max. effect (%)	No effects	No effects*
	EC_{50}(%) (95% CI)	ND	ND
***L. minor* growth rate inhibition (frond number)**			
CWE	Max. effect (%)	100 at 50%	58.16 at 100%
	EC_{50}(%) (95% CI)	13.16 ± 0.89 (11.26 – 15.06)	42.32 ± 7.10 (27.17 – 57.46)
***D. magna* immobilization**			
CWE	Max. effect (%)	100.00 at 12,5%	100.00 at 25%
	EC_{50}(%) (95% CI)	ND	ND

ND = not determined; *stimulation compared to the MBL control was found for all higher concentrations of CWE samples.

R. subcapitata test results show that both untreated (t= 0 days) and biotreated (t= 7 days) CWE did not induce any inhibition in the growth rate of this microalgae but rather stimulation, as higher concentrations achieved higher cells densities. Thus, *R. subcapitata* is likely taking advantage of the nutritional load of the effluent and eventually of the by-products excreted by the Asian clam in the end of the biotreatment to grow, instead of being affected by the toxicity of CWE as initially expected.

The untreated CWE (day 0) was lethal to *D. magna* in all tested concentrations (100, 50, 25 and 12.5%), as the observed immobilisation achieved 100% in all cases. In relation to the biotreated CWE (day 7), *D. magna* also achieved a mortality of 100% at the highest tested concentrations (100, 50 and 25%), but the smallest concentration (12.5%) allowed the survival of 40% of the tested organisms, denoting that the biotreatment reduced the toxicity of CWE to *D. magna*.

The most remarkable results were achieved with *L. minor*, as it showed less than 60% growth rate inhibition based on frond number following exposure to full-strength biotreated CWE (day 7; Table 2 and Figure 3.7) compared to full inhibition obtained following exposure to 50% untreated CWE. The corresponding EC50 estimates confirm this pattern, supporting the capacity of the Asian clam to decrease the toxicity of CWE. Figure 3.8 clearly illustrates the improvement achieved by the biotreatment: the fronds of *L. minor* were white/yellow and contained fungi after being exposed to untreated CWE (t = 0 days, Figure 3.8A). However, when exposed to the same concentrations of the biotreated CWE (t = 7 days), fronds appear green and healthy (Figure 3.8B).

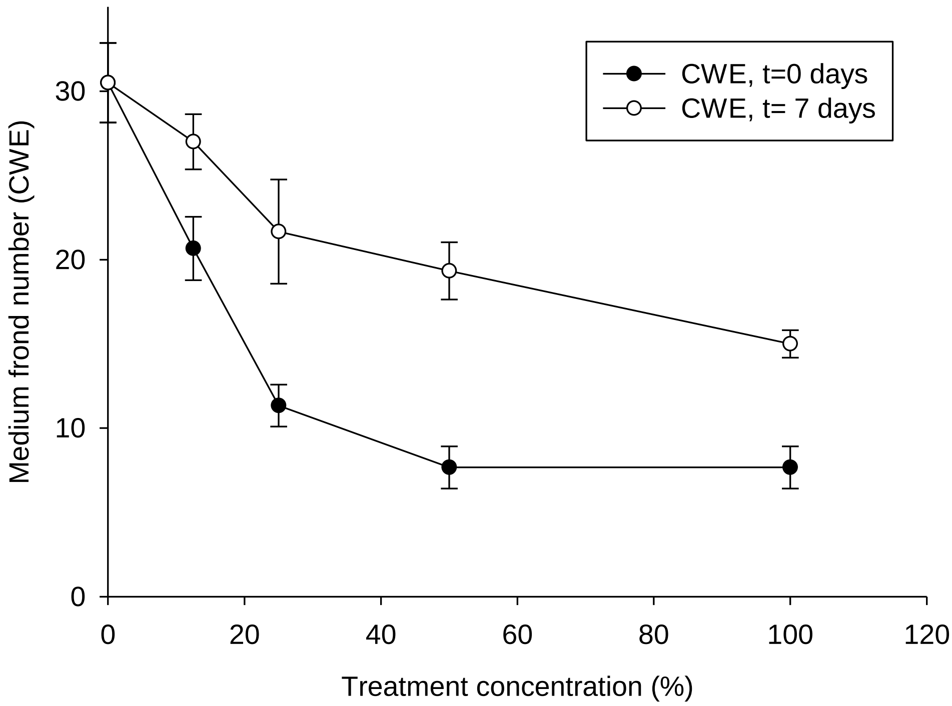

Figure 3.7. Frond number variation on *L. minor* following exposure to untreated (day 0) and biotreated (day 7) CWE.

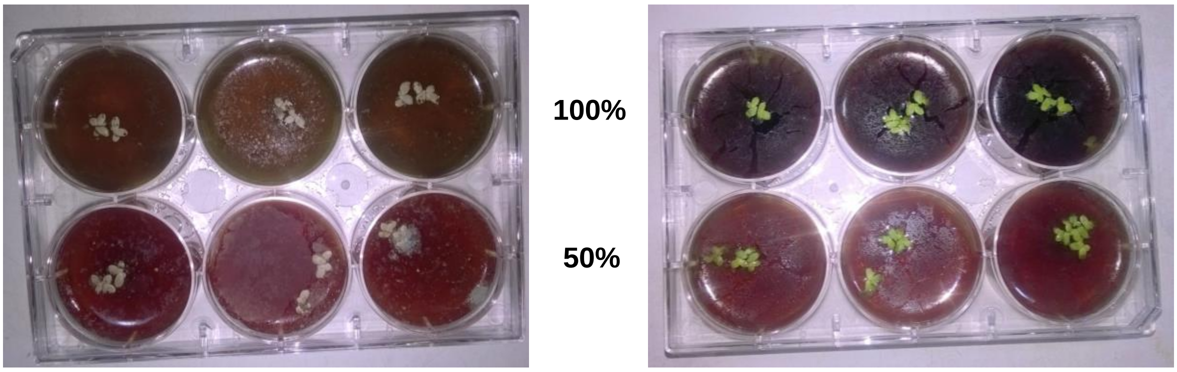

Figure 3.8. Images of the end of the experiment of the *L. minor* growth inhibition test exposed to CWE, at the concentrations of 100% and 50%: A- CWE (t= 0 days); B –CWE (t= 7 days).

Ultimately, these results show the ability of the Asian clam in reducing the inherent toxicity of CWE, further supporting the chemical dynamics among the aqueous media, the soft tissues and the shells. The use of laboratory bioassays with standard species is of utmost importance for the quantification of the toxicological effects caused by chemical contaminants burdening complex mixtures such as CWE. Our results overall show that the joint chemical and ecotoxicological assessment is advantageous to better understand the efficacy of remediation strategies targeted at pollutant effluents and the environmental hazardous potential of both the untreated and treated effluents. It is the integration of both strategies that enables a more robust conclusion on the final water quality improvement, and hence provides a more holistic scenario (Connon et al. 2012).

Indeed, biofiltration is suggested to be included as a tertiary treatment in wastewater treatment plants (Gomes, Matos, et al. 2018; Magni et al. 2015; Pipolo et al. 2017), particularly in the case of olive oil wastewaters and their ongoing lack of a proper treatment strategy. Our results represent a further step in this process, as they show that the Asian clam was in fact capable of reduce the toxicity of CWE. Meanwhile, there should be an extreme care in the application of invasive species like the Asian clam in any treatment settings that may have any connection with natural aquatic ecosystems, unless these are already invaded (Gomes, Matos, et al. 2018; Elliott et al. 2008). Notwithstanding, not all clams have survived in the end of the biotreatment, which means that the inherent toxicity of CWE could compromise the reproduction of the clams and, with that, reduce the dispersal risks posed by this pest in an industrial environment. Nonetheless, this assumption lacks additional research.

At an industrial level, the use of bivalves in wastewaters decontamination systems could bring up the problem of their collection costs. However, if appropriately managed for example in parallel with pest management programmes envisaging clam's mechanical removal from invaded sites or infested industrial settings, a positive economic balance could be achieved. Furthermore, depending on future confirmation of the efficacy of the clams as integrated in wastewater treatment systems, this approach constitutes a way of intensifying the removal of this species from the environment, and a cost-effective solution to industries that already need to resort periodic removals of the Asian clam from their pipes. The final destiny of the contaminated organisms following treatment service represents a last challenge (and environmental concern) when discussing the use of these animals in bioremediation processes. Literature already refers landfills and incineration as the main management solutions applied to wastewater treatment by-products (Magni et al. 2015; Gomes, Matos, et al. 2018; Pipolo et al. 2017), as these are the strategies used for sewage sludge; yet, aerobic digestion has also been referred as another suitable option (Gomes, Matos, et al. 2018). Also, shells exposed to CWE can be applied as fertilizers and soil correction agents (i.e. pH increase and reduction of acidic inputs) due to the organic and vegetable nature of this wastewater (Yao et al. 2014).

3.4. Conclusions and future perspectives

This works presents the first approach linking the invasive bivalve *C. fluminea* with the treatment of olive oil wastewaters, and our results showed that this organism presents a promising biological tool to reduce the chemical burden and toxicity of these wastewaters.
In the future, there is still much to explore regarding the improvement of the biofiltration capacity of the Asian clam over OOMW, focusing on both soft tissues and shells. At last, the optimization of an industrial-scale configuration system incorporating the Asian clam, aimed at the olive oil industry should also be considered in following studies.

3.5. Acknowledgments

The authors would like to thank to AOTAD, “Associação dos Olivicultores de Trás-os-Montes e Alto Douro” for their fundamental role in providing us the olive oil wastewaters necessary to accomplish this work. We are also grateful to all colleagues that helped in the field work for the collection of the clams.

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3.7. Supplementary material

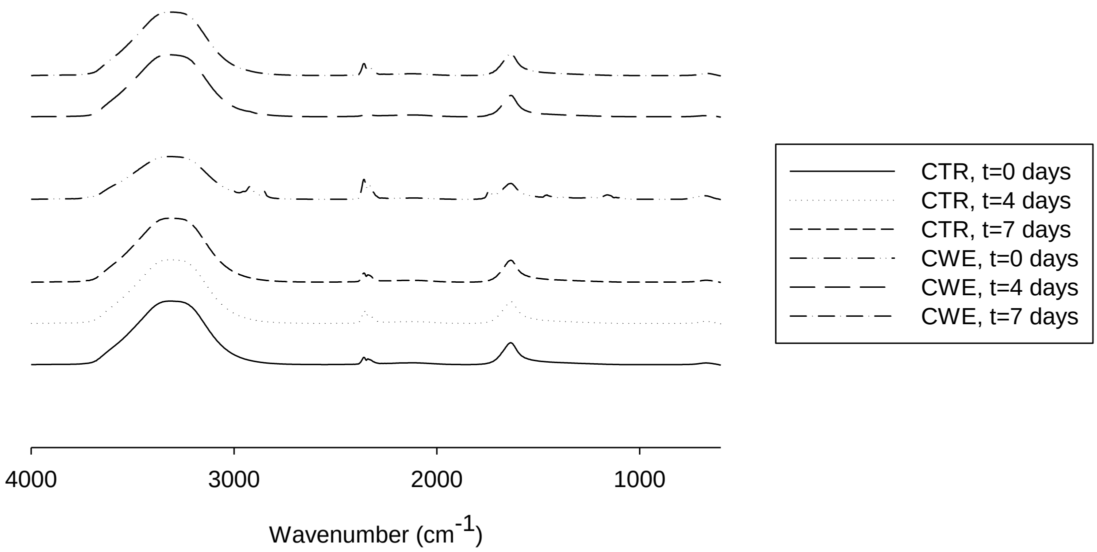

Figure S1. ATR-FTIR spectra in the region of 4000-600 cm^{-1} of both CTR and CWE, before and after 7-days exposure for filtration by *C. fluminea*. The spectra reflect the organic variation in both samples.

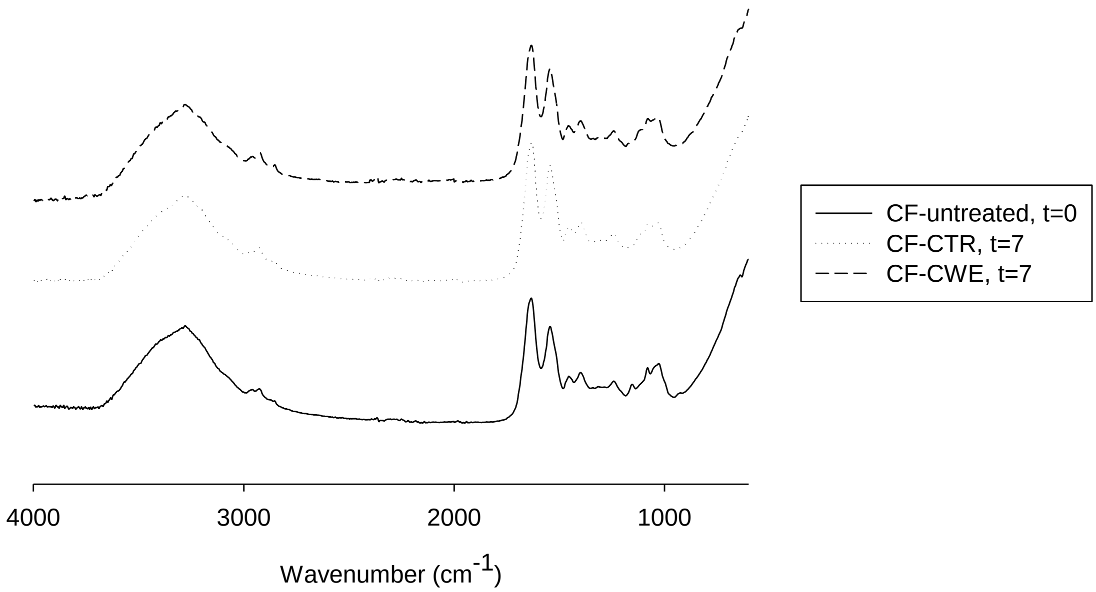

Figure S2. ATR-FTIR spectra in the region of 4000-600 cm^{-1} of both CTR and CWE, before and after 7-days exposure for filtration by *C. fluminea*. The spectra reflect the organic variation in the soft tissues of the Asian clam.

Table S1 – Absorbance readings reflecting aromatic compounds (270 nm), coloured dissolved organic carbon (320 nm) and coloured compounds (465 nm), recorded for CTR and CWE treatments at day 0 and day 7, respectively.

Treatment		**Absorbance (270 nm)**	**Absorbance (320 nm)**	**Absorbance (465 nm)**
CTR	Day 0	0.00100	0.990	0.00
	Day 3	0.140	18.1	0.0300
	Day 7	1.14	3.23	0.840
CWE	Day 0	2.80	672	2.88
	Day 3	4.00	721	4.00
	Day 7	4.00	644	4.00

Table S2 – Statistical analysis (two-tailed paired-sample t tests) applied to the CTR or CWE to compare between t= 0 days and t= 7 days.

Wavenumber (cm^{-1})	**Compared Samples**	**Statistical Analysis (two-tailed paired-sample t tests)**
3650-3000	CTR (t= 0 days) – CTR (t= 7 days)	$t_{0.05(2),2}$= -1.63 p= 0.245
	CWE (t= 0 days) – CWE (t= 7 days)	$t_{0.05(2),2}$= 6.82 p= 0.0208
3000-2810	CTR (t= 0 days) – CTR (t= 7 days)	$t_{0.05(2),2}$= -1.45 p= 0.283
	CWE (t= 0 days) – CWE (t= 7 days)	$t_{0.05(2),2}$= 12.9 p= 0.00593
1750-1180	CTR (t= 0 days) – CTR (t= 7 days)	$t_{0.05(2),2}$= -1.71 p= 0.229
	CWE (t= 0 days) – CWE (t= 7 days)	$t_{0.05(2),2}$= 7.89 p= 0.0257
1180-900	CTR (t= 0 days) – CTR (t= 7 days)	$t_{0.05(2),2}$= -0.247 p= 0.827
	CWE (t= 0 days) – CWE (t= 7 days)	$t_{0.05(2),2}$= 2.63 p= 0.119

Table S3 – Statistical summary of two-tailed paired-sample t tests comparing the peak areas found for soft tissues at the beginning of the experiment (t = 0 days) and those found in soft tissues exposed to CTR and CWE at the end of the experiment (t = 7 days).

Wavenumber (cm^{-1})	**Compared soft tissues samples**	**Statistical Analysis (two-tailed paired-sample t tests)**
3650-3000	CTR (t= 0 days) – CTR (t= 7 days)	$t_{0.05(2),2}$= 0.00467 p= 0.997
	CTR (t= 0 days) – CWE (t= 7 days)	$t_{0.05(2),2}$= -0.588 p= 0.616
3000-2810	CTR (t= 0 days) – CTR (t= 7 days)	$t_{0.05(2),2}$= 0.0819 p= 0.942
	CTR (t= 0 days) – CWE (t= 7 days)	$t_{0.05(2),2}$= 0.323 p= 0.778
1750-1180	CTR (t= 0 days) – CTR (t= 7 days)	$t_{0.05(2),2}$= -0.426 p= 0.712
	CTR (t= 0 days) – CWE (t= 7 days)	$t_{0.05(2),2}$= -0.814 p= 0.501
1180-900	CTR (t= 0 days) – CTR (t= 7 days)	$t_{0.05(2),2}$= -0.144 p= 0.899
	CTR (t= 0 days) – CWE (t= 7 days)	$t_{0.05(2),2}$= -0.171 p= 0.880

Table S4 – Statistical summary for two-tailed simple t tests addressing the differences between variation in peak areas through the experiment in soft tissues from the CTR and CWE treatments.

Wavenumber (cm^{-1})	**Compared soft tissues samples**	**Statistical Analysis (two-tailed simple t tests)**
3650-3000	CTR (t= 7 days) – CWE (t= 7 days)	$t_{0.05(2),2}$= 1.42 p= 0.251
3000-2810	CTR (t= 7 days) – CWE (t= 7 days)	$t_{0.05(2),2}$= -0.930 p= 0.421
1750-1180	CTR (t= 7 days) – CWE (t= 7 days)	$t_{0.05(2),2}$= -3.66 p= 0.0352
1180-900	CTR (t= 7 days) – CWE (t= 7 days)	$t_{0.05(2),2}$= -1.94 p= 0.147

Table S5 – Statistical summary of two-tailed paired-sample t tests comparing the peak areas found for shells at the beginning of the experiment (t = 0 days) and those found in shells exposed to CTR and CWE at the end of the experiment (t = 7 days).

Wavenumber (cm^{-1})	**Compared shell samples**	**Statistical Analysis (two-tailed paired-sample t tests)**
3650-3000	CTR (t= 0 days) – CTR (t= 7 days)	$t_{0.05(2),2}$= -1.90 p= 0.308
	CTR (t= 0 days) – CWE (t= 7 days)	$t_{0.05(2),2}$= -0.535 p= 0.687
3000-2810	CTR (t= 0 days) – CTR (t= 7 days)	$t_{0.05(2),2}$= 4.94 p= 0.127
	CTR (t= 0 days) – CWE (t= 7 days)	$t_{0.05(2),2}$= -1.26 p= 0.427
1750-1180	CTR (t= 0 days) – CTR (t= 7 days)	$t_{0.05(2),2}$= 28.7 p= 0.0221
	CTR (t= 0 days) – CWE (t= 7 days)	$t_{0.05(2),2}$= -1.49 p= 0.376
1180-900	CTR (t= 0 days) – CTR (t= 7 days)	$t_{0.05(2),2}$= 4.05 p= 0.154
	CTR (t= 0 days) – CWE (t= 7 days)	$t_{0.05(2),2}$= -1.34 p= 0.407

Table S6 – Statistical summary for two-tailed simple t tests addressing the differences between variation in peak areas through the experiment in shells from the CTR and CWE treatments.

Wavenumber (cm^{-1})	**Compared shell samples**	**Statistical Analysis (two-tailed simple t tests)**
3650-3000	CTR (t= 7 days) – CWE (t= 7 days)	$t_{0.05(2),2}$= -0.204 p= 0.857
3000-2810	CTR (t= 7 days) – CWE (t= 7 days)	$t_{0.05(2),2}$= -1.53 p= 0.265
1750-1180	CTR (t= 7 days) – CWE (t= 7 days)	$t_{0.05(2),2}$= -1.64 p= 0.243
1180-900	CTR (t= 7 days) – CWE (t= 7 days)	$t_{0.05(2),2}$= -1.59 p= 0.253

CHAPTER 4. Biosorption potential of the shell of *Corbicula fluminea* towards olive oil mill wastewaters

Abstract

Olive oil mill wastewaters (OOMW) are one of the most toxic industrial wastewaters, widely produced and discharged in the Mediterranean basin. Its recalcitrant phenolic components, along with its intense seasonal production and lack of proper treatment solutions render this wastewater an ongoing environmental problem. In this context, bioremediation has been raising interest due to its environmentally friendlier nature and cost-efficiency, compared with other physicochemical approaches. The Asian clam, *Corbicula fluminea,* is a widely invasive freshwater bivalve, and in this study the biosorption capabilities of its shells regarding OOMW were explored. Shells were exposed to OOMW and, at the end of the biotreatment, untreated and biotreated fractions of the wastewater were compared and analysed in terms of their chemical composition. Ultimately, this study showed that the shells of *C. fluminea* as an isolated matrix are not a suitable recipient for the organic components present in OOMW.

Keywords: *Corbicula fluminea*; olive oil mill wastewater; bioremediation; biosorption; shells

4.1. Introduction

Literature regarding the chemical composition of bivalve shells is scarce, and so are studies specifically focused on *C. fluminea*. Thereby, some of the information presented here is generalized to the Bivalvia class. Organisms within this class possess a shell composed mainly by calcium carbonate (approximately 90% by mass) and an organic matrix (approximately 0.01 to 5% by mass) (Suzuki & Nagasawa 2013). Calcium carbonate is commonly present in the form of aragonite and calcite crystals (alone or mixed) that are organized into three main microstructures: (i) nacreous and prismatic; (ii) foliated; and (iii) crossed lamellar layers (Kobayashi & Samata 2006).

Regarding organic components, the shell compartment is linked to the biological matrix through a ligament (or resilium) - composed internally of aragonite fibers and externally of fibrous glycoproteins, this component plays an important role for the mechanical movements of the valves (Paula & Silveira 2009). The adductor muscle is located in the mantle cavity and it is also part of the hinge mechanism of bivalves (Cadée 2002). The external layer of the molluscan shells (*periostracum*) is also a partially organic component of the shell matrix, responsible for organizing shell layers particularly in the prismatic microstructure (Paula & Silveira 2009; Marin et al. 2012; Suzuki & Nagasawa 2013).

Bivalves are known as ecological engineers mainly due to their high production of shells, these being their longer-living components, with a notorious role on the physical structure of the benthic compartment (Ilarri et al. 2018; Gutiérrez et al. 2003). In fact, the importance of bivalve shells in benthic habitats is remarkable since they entail heterogeneity, complexity and affect structural processes (Gutiérrez et al. 2003; Ilarri et al. 2014). However, excessive deposition of shells as it is often the case regarding the invasive Asian clam, or other invasive bivalves, could deeply transform the habitat in the long-term through its homogenization, with severe impacts on the establishment of other species and subsequently on the diversity and traits of the communities (Ilarri et al. 2018; Gutiérrez et al. 2003).

In freshwater systems, shell's decay rates are clearly impacted by water chemistry, currents and mechanical abrasion, although the effects of these abiotic factors depend on several characteristics of the shells: size (of the shell and of its calcium carbonate crystals), chemical composition and organic matter structure (Strayer & Malcom 2007). Water hardness is also a crucial parameter – hard waters allow the sequestration of calcium carbonate by their bivalve populations contrarily to soft waters. In the case of waters saturated with calcium carbonate, chemical dissolution is the rate-limiting step for shell decay, as the organic matter will be decomposed by the microbial communities and these processes will make shells more available to physical forces, such as water currents (Strayer & Malcom 2007). Besides, the decay rates of the shells are higher in aquatic than terrestrial systems due to the mechanical action of the waters and the consequent faster dissolution rates, which also increase the rates at which minerals become bioavailable (Ilarri et al. 2015). Although the production of shells is higher in aquatic habitats, in extreme scenarios such as floods and droughts, a large quantity of shells can be transferred to terrestrial systems – therein, as shells take longer to decay, their inputs persist for longer than in aquatic ecosystems (Novais et al. 2015), essentially meaning that calcium ions will be available and biogeochemical cycles may experience some modifications through time (Ilarri et al. 2015).

The potential use of shells of *C. fluminea* in remediation processes targeting organic compounds is not widely investigated until now (Silva et al. 2016). However, there are some studies exploring this potential valuation approach with metal contaminants. For example, in the study by Rosa et al. (2014), the shells of the Asian clam were found to be the major recipients of metal ions originated from acid-mine drainage effluents compared to soft tissues following filtration. Other studies also evidenced the role of shells in the bioaccumulation of metals, some of them highlighting their importance in paleontological studies (Zhao et al. 2017; Merschel & Bau 2015). Indeed, biomineralization is a well-known process performed by a plethora of molluscs including bivalves, that can be understood as a protection strategy of the individual to cope with the xenobiotic exposure – by deposing metals in the shell, bivalves are actively separating them from their organic tissues, which means reduced requirements of metabolization and/or detoxification (Gifford et al. 2004). Metals can be either effectively incorporated in shells, or they can substitute calcium ions in the shell structure if their ionic radius is dimensionally comparable to calcium (Gifford et al. 2004). Biomineralization studies were already performed with promising results using oysters for bioremediation in the aquaculture sector (Gifford et al. 2004), and also to purify domestic wastewaters (Shih & Chang 2015). While the previous studies focused on using the shells of living organism (thus, considering the biomineralization process), the shells of *Mytilus edulis* per se were proven to be a successful sorbent in removing textile dyes from industrial wastewaters (Maghri et al. 2012).

Although the biomineralization studies available so far provide good perspectives for the use of bivalves as bioremediation agents, they all consider the use of living organisms.

The use of living bivalves is indeed appealing since both accumulation in soft tissues and mineralization may concur to achieve better levels of efficacy in the removal of contaminants from water. However, when invasive biofouling bivalves such as *C. fluminea* are considered as providers of these services, the use of living organisms is challenged by the need to prevent dispersal and deleterious impacts in natural communities. In this context, we hypothesised that the periostracum of shells of *C. fluminea* has an organic composition that allows the collection of waterborne organic contaminants through sorption. The present study hence intends to evaluate the capacity of *C. fluminea* shells as a sorption material following exposure to olive oil wastewater (OOMW), herein used as a model for a wastewater matrix burdened by organic, highly toxic compounds. The success of this approach could potentially present a sustainable bioremediation solution itself, not only as a possible treatment step in wastewater treatment plants but also because it doesn't pose the dispersion risks entailed by living organisms.

4.2. Materials and Methods

4.2.1. Wastewater Sampling

Wastewater samples were provided by the regional olive oil producers association (AOTAD) from Mirandela, North-East Portugal. The samples corresponded to the final fraction of the three-phase extraction process of olive oil**.** Samples were collected after the olive oil extraction, transported to the laboratory and stored at 20°C until used in the experiments.

4.2.2. Test Organisms

C. fluminea individuals were collected in Casal de São Tomé, Mira, Portugal (40°25'06.90''N, 8°44'13.18''W). Locally, clams were chosen according to their shell length (between 18 and 30 mm). These individuals were transported to the laboratory along with a sample of the local water. They were kept for a two-week quarantine period, gradually acclimating to the laboratory conditions: clams were firstly kept with equal proportions of dechlorinated municipal water and local field water, and then the later was progressively replaced by dechlorinated water – twice a week the water was fully renewed. Also, the clams were kept with continuous aeration, temperature (20 ± 2°C) and photoperiod (16h light: 8h dark) and were fed *ad libitum* with *Raphidocelis subcapitata* suspensions twice a week. Before the onset of the experiment, shells were cleared of soft tissues and preserved at -20°C.

4.2.3. Biotreatment Experiment

Biotreatment experiment using thawed shells of *C. fluminea* was conducted for 14 days. Preliminary assays were carried out to define the lowest practical dilution level for exposing the shells: the tested OOMW was solid, thus we could neither guaranty a homogeneous exposure of the whole shell surface nor a homogeneous clearing of non-absorbed residues from shells undergoing chemical analysis at the end of the assay. Therefore, a 60% OOMW dilution in dechlorinated municipal water was used as the test treatment. Three treatments were set in this experiment: a control (CTR) composed of blank dechlorinated municipal water added shells (30 per replicate, 68.05 g), which allowed monitoring any potential release of chemicals from the shell matrix into the water; a reference treatment (REF) composed of 100% OOMW with no shells added for monitoring on changes in chemical composition of the OOMW during the exposure period; and a treatment composed of 60% OOMW added shells (30 per replicate, 70.66 g). Glass vessels were filled with 500 mL of each test solution and were kept under continuous agitation (orbital shaker), constant temperature (20 ± 2°C) and photoperiod (16h light: 8h dark) through the whole biotreatment experiment. A total of three replicates were established per treatment. Baseline water quality parameters (pH, temperature and conductivity) were measured at the beginning and the end of the experiment with a multiparameter probe (Aquaprobe® AP-2000). Samples of the test medium were also collected following this timeline and preserved at -20°C until further chemical analyses (see section 4.1.4). In addition, *C. fluminea* shells were collected at the beginning (sub-sample of the batch that was used to perform the experiment) and at the end of the experiment. Shells were dried at room temperature and then preserved at -20°C for chemical analysis.

4.2.4. Chemical Analyses

Colorimetric methods (Shimadzu UV-1800, UV Spectrophotometer) were used to quantify aromatic and coloured compounds (270 nm and 465 nm, respectively) and coloured dissolved organic carbon (CDOC; 320 nm) of OOMW, at the beginning and end of the biotreatment. Chemical oxygen demand (COD) concentrations were measured at the same time points (Hanna Instruments 93754C – high range: 0-15000 mg/L; multiparameter bench photometer C214), following the USEPA method 410.4 (USEPA 1993).

OOMW and shells of *C. fluminea* were analysed in samples taken at the beginning and the end of the test. Fourier-transform infrared spectroscopy with attenuated total reflectance (ATR-FTIR) analyses were carried out using a Perkin Elmer (USA) Spectrum BX FTIR instrument, with a resolution of 4 cm^{-1} within the 4000 - 500 cm^{-1} range. Air was used for the background spectrum.

4.2.5. Data Analyses

Alterations in the organic composition of OOMW were analysed through paired t-tests comparing between its initial (t = 0 days) and final (t = 14 days) molecular composition considering peak areas of spectra within each ATR-FTIR region (3650-3000; 3000-2810; 1750-1180; 1180-900 cm^{-1}) for both the CTR, REF and OOMW. Variation in the molecular composition of the shells was determined based on the difference in peak areas recorded for day 14 and day 0. This variable was used for graphical analysis purposes. A simple t-test was used to statistically compare the molecular composition of shells tested against CTR and OOMW (t = 14 days).

All analyses were performed using the Microsoft Office 365 Excel. A significance level of 0.05 was considered in all statistical analyses.

4.3. Results and Discussion

4.3.1. Chemical characterisation of the wastewater

The biotreatment experiment showed that baseline parameters were similar to those ones recorded with the centrifuge washing effluent in the biofiltration experiment (see chapter 3 in this Dissertation). The temperature in the test solutions was constant throughout the experiment in CTR and OOMW treatment samples (t = 0 days: 20.65 ± 0.35 ºC, t = 14 days: 20.33 ± 1.63 ºC; n = 3). pH was constant and similar in all CTR samples (t = 0 days: 7.19 ± 0.016; t = 14 days: 7.58 ± 0.036; n =3); whereas in OOMW pH was clearly acidic in the beginning (4.06 ± 0.069; n = 3), and then slightly increased until the end of the experiment (5.24 ± 0.037; n = 3). These pH results for OOMW are commonly reported in other studies (Aggoun et al. 2016; Dermeche et al. 2013; Muktadirul Bari Chowdhury et al. 2013) and can be easily explained by the oxidation of phenolic compounds, amongst other organic compounds (Muktadirul Bari Chowdhury et al. 2013). Regarding conductivity, there was a slight increase in the CTR from day 0 (237.67 ± 4.78 µS/cm; n = 3) until the end (440.67 ± 16.05 µS/cm; n = 3); and an increase in OOMW from day 0 (7636.67 ± 310.26 µS/cm; n=3) to day 14 (9300.67 ± 314.17 µS/cm; n=3), and this could be explained by the condensation of soluble salts through the experimental period (Muktadirul Bari Chowdhury et al. 2013). Absorbance at 270 nm (Table S1) remained constant and similar throughout the experiment, for both REF and OOMW, suggesting no overall changes in the aromatic compounds' composition neither between REF and OOMW, nor between the untreated and the treated OOMW. The same results were evident for CDOC (320 nm, Table S1). Absorbance at 465 nm (characteristic of coloured compounds, Table S1) was also constant and similar throughout the assay for REF and OOMW samples, which reinforces their similarity.

Chemical oxygen demand (COD) was analysed in REF and OOMW samples, in the beginning (day 0) and end (day 14) of the biotreatment (Table 1). Initially, the COD level of the REF treatment (100%) was higher comparing with the level obtained in the OOMW treatment (60%). However, in the end of the biotreatment the situation inverts, as COD was higher in the OOMW than in the REF treatment. It was expected that the shells could be able to incorporate or adsorb some organic matter into its structure, thus reducing the COD content of the aqueous phase throughout the experiment; instead, they may have added more organics to the wastewater causing an increase in the COD. On the other hand, the chemical reactions may have occurred in the REF treatment can justify the decrease in COD to some extent; and the reason why the same reactions did not occur in OOMW could be related with the induction of anoxic zones by the shells, leading to the decreased of oxidation processes. Thus, the biotreatment was clearly unsuccessful regarding this parameter, and all COD final levels were not within the legal limit established for discharge in natural aquatic systems (150 mg/L O_2 according to the Law-by-Decree no. 236/98 of August 1, 1998).

Table 1 – COD characterisation of REF and OOMW before and after the biotreatment experiment with shells of *C. fluminea* (average between two replicates).

Sample	COD (g/L)
REF (day 0)	655 ± 185
OOMW untreated (day 0)	420 ± 130
REF (day 14)	435 ± 195
OOMW biotreated (day 14)	580 ± 80

ATR-FTIR results from CTR, REF and OOMW treatments are presented in Figure 4.1. Starting with the region within 3650-3000 cm^{-1}, CTR peaks are very high in both time periods, which probably relates to the presence of water in the test solution (OH stretching vibrations are read in this region). This hypothesis is corroborated by the peaks of lower intensity found for the REF, which was monitored undiluted through the experiment, thus bearing lower water content. In the REF treatment, there is a small increase from day 0 to day 14 that could be related with chemical degradation of the phenolic compounds present in this matrix. The areas of OOMW present the same order of magnitude as the CTR treatment, but it is likely that in the case of OOMW the peaks relate to both water and phenolic compounds (OH stretching vibrations) typical of these effluents (Aggoun et al. 2016; Obied et al. 2005). No statistical differences between day 0 and day 14 were noticed in any of the treatments (Table S2).

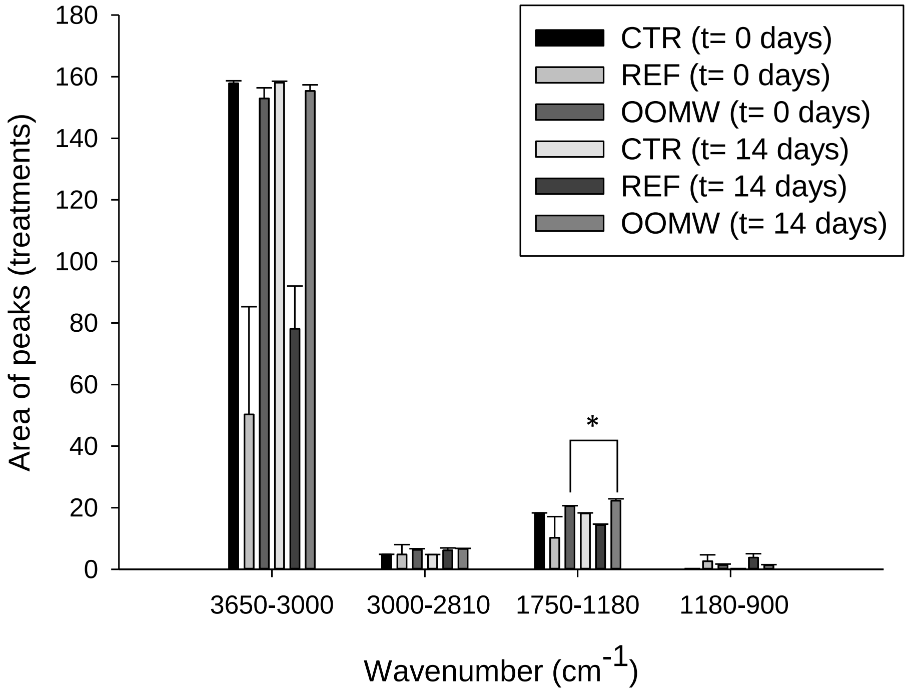

Figure 4.1. Mean areas for the different regions of the ATR-FTIR spectra regarding the CTR, REF and OOMW treatments, through a 14-day filtration period. Each peak area results from n = 3 replicates sampled and separately read per treatment. Error bars represent the standard deviation. The asterisk denotes a significant variation occurring in the molecular composition of the OOMW through the experiment (paired-sample t-test comparing T0 with T14; $p < 0.05$).

The region between 3000 and 2810 cm^{-1} is characterized by the presence of aliphatic compounds (lipids, waxes and long-chain structures). Taking this into consideration, it was expected that only residual traces would be found for CTR in this region, probably associated with polysaccharides released by the shells structure (*periostracum*) (Marin & Luquet 2004). Low levels in the CTR are clear from the reading of Figure 4.1, but these did not increase significantly through the experiment (Table S2), which suggests that the release suggested in the literature did not occur in this case. Also, similar low and largely invariable (no significant differences were found through the experiment; Table S2) levels were found for the other two treatments, suggesting that the burden of the wastewater in waxes and hydrocarbons (olives skin), or lignin, cellulose, hemicellulose and glycans (olive stones), as well as and fatty acids and carbohydrates from the residual oil pulp (Dashti et al. 2015; La Rubia-García et al. 2012; El-Abbassi et al. 2012), was not affected by the presence of the shells.

The presence of aromatic compounds (C=C), amides (C=N), ketones and aldehydes (C=O) stretching vibrations can be inferred from the peaks found within the 1750-1180 cm^{-1} region of the spectra. No feasible conclusions can be taken regarding this region since the read levels were very similar in all treatments for both time points including in the CTR (note that CTR readings at t = 0 days correspond to dechlorinated municipal water). This pattern was somewhat unexpected since the presence of aromatic structures is typical in OOMW as reported in the literature (El-Abbassi et al. 2012; Justino et al. 2012). A slight increase in the peak area from the beginning to the end of the experiment can be noticed in OOMW that it is significant (Table S2). The input of these types of organic groups is probably originated from the dissolution of part of the organic structure of the shell itself, releasing amides present in biogenic carbonates (Suzuki & Nagasawa 2013; Dauphin & Denis 2000).

At last, residual traces of CH_2 and -C-O groups (1180-900 cm^{-1} region) were found in REF and OOMW, which is consistent with the presence of proteins and amino acids as reported to be typical in the literature for these effluents (Morillo et al. 2009), while no signals were detected for the CTR samples within this wavenumber range.

4.3.2. Chemical characterisation of the shell of *C. fluminea*

ATR-FTIR spectra of the shells are presented in Figure S2 and corresponding areas were used to calculate the variation occurring during the 14 days of the experiment for each spectra region as shown in Figure 4.2.

Shells belonging to the CTR treatment (t=14 days) present a significantly lower variation (Table S4) compared to OOMW within 3650-3000 cm^{-1}, but in both cases there seems to have been a relevant loss of phenolic and other related compounds from the shells into the media rather than the desirable accumulation of such compounds in the shell. Lipids and polysaccharides, amongst other chemical compounds, are part of the organic phase of mollusc shells (Suzuki & Nagasawa 2013). However, the corresponding FTIR region (3000-2810 cm^{-1}) denotes peaks of low intensity at the beginning of the experiment, which suggests that the tested shell samples were not rich in these compounds. The few possibly existent in these shells were lost into the OOMW as indicated by the negative variation found in this FTIR region, a pattern that was opposite to that recorded in the CTR (the treatments were found significantly different in this context; Table S4). The aragonite structure of the shell reflects in some characteristic peaks in the 1750-1180 cm^{-1} region (Dauphin & Denis 2000), as evidenced by the corresponding area at the beginning of the experiment and reinforced by the increase observed at day 14 in the CTR. The partial dissolution of aragonite could be explained by the high levels of calcium ions registered in the municipal waters of Aveiro (21 mg/L of Ca as indicated by the municipal water supply services), favouring the formation of aragonite crystals throughout 14 days of biotreatment. On the contrary, shells exposed to OOMW experienced a small decrease in these peak areas through the experiment (the reads from CTR and OOMW were confirmed to be significantly different; Table S4) that could be related to the increase dissolution of aragonite in the wastewater throughout the experience. At last, stretching bonds from -C-O and CH_2 associated to the region within 1180-900 cm^{-1} neither varied significantly through the test in any treatment (Table S3) nor resulted in significantly different areas following exposure between CTR and OOMW (Table S4).

T = 0 70.7 ± 51.4 2.81 ± 1.56 9.49 ± 6.27 0.53 ± 0.23

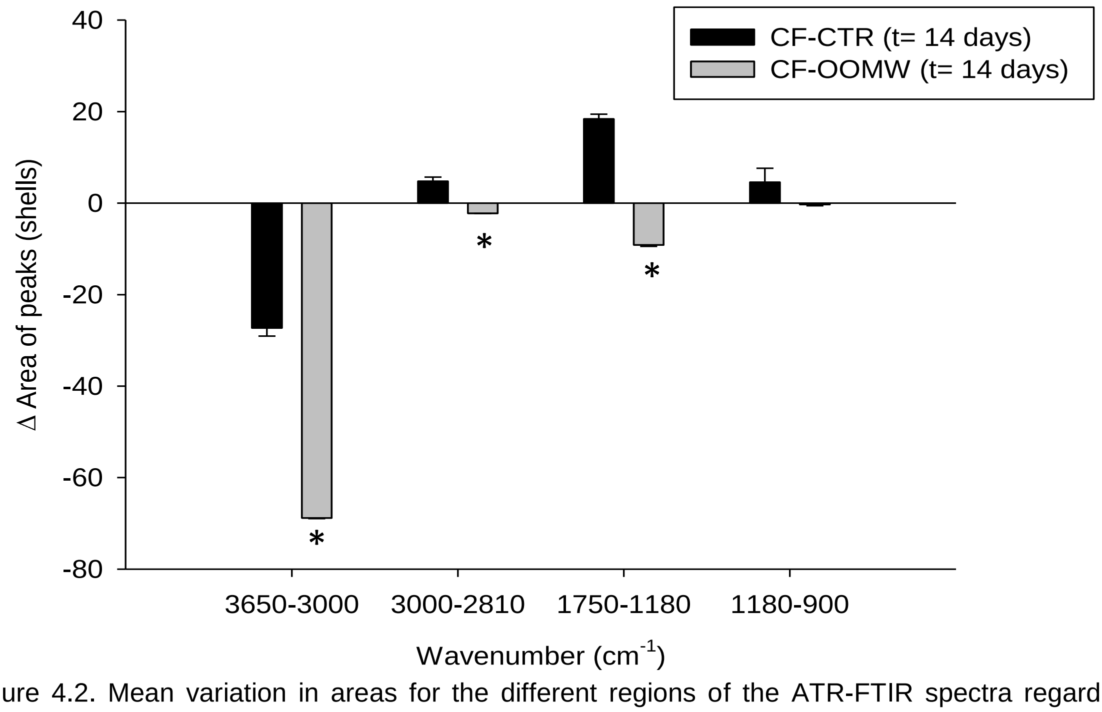

Figure 4.2. Mean variation in areas for the different regions of the ATR-FTIR spectra regarding shells of *C. fluminea* through a 14-day filtration period over the CTR and the OOMW in the biotreatment experiment. Readings at the beginning of the experiment served as the baseline to calculate the variation in peak area for each region, i.e. each record (n = 3 per treatment) obtained at the end of the experiment was subtracted the corresponding average record obtained for samples taken at the beginning of the experiment. Error bars represent the standard deviation. Asterisks denote significant differences between shells exposed to CTR and OOMW regarding the final areas reading (simple t-test; $p < 0.05$). In the top of the plot, the average areas calculated for each region at the beginning of the experiment in shells are given for reference purposes.

Overall, our results do not show any evidences supporting the hypothesis that there is a sorption potential of the shells of the Asian clam towards organic compounds, namely the ones present in the OOMW composition. Moreover, pH levels from the OOMW treatment experiences an increase from day 0 to day 14, which supports that not only the shells were not capable of adsorbing organic compounds, but they may have also been dissolving into OOMW.

The importance of shells from invasive, widely established bivalves such as the Asian clam, as a adsorbent alternatives lies on the fact that they are naturally abundant resources posing no hazardous issues, available at low-cost (Ismail et al. 2014). Indeed, it has been shown that shells from several living molluscs have a high affinity with metallic ions (Rosa et al. 2014; Huanxin et al. 2000; Liu et al. 2009; Du et al. 2011; Ismail et al. 2014) as they are capable of undergoing ion exchanges by substituting calcium cations with other metallic ions present in the test solution (Tudor et al. 2006). Although the shells *per se* (i.e. with no living organism regulating or mediating the processes) have been already proven a capacity as biosorbents of industrial wastewaters (Maghri et al. 2012), the present study did not support this potential for OOMW.

Shells of invasive bivalves can constitute a more powerful resource comparing to the living organism, in the sense that they do not entail the environmental threat represented by the dispersion of a nuisance species during a less controlled treatment action. Besides assessing the suitability of *C. fluminea* shells as biosorbent materials targeting wastewaters other than OOMW, there is a plethora of applications that are worth exploring using bivalve shells. For instance, calcium carbonate is presently used in agriculture, for example to recover the buffer capacity of soil, increase soil pH, reduce acidic inputs and improve the nutritional components of the soil, i.e. phosphates availability and content of organic matter (Yao et al. 2014; Morris et al. 2018). Therefore, it is important to continue investigating this material towards other bioremediation applications.

4.4. Conclusions and future perspectives

This work is the first approach addressing the potential of shells of the invasive bivalve *C. fluminea* for the treatment of olive oil mill wastewaters, presenting an initial set of data regarding their success as absorbents towards organic compounds. Our results do not support the use of shells to specifically remediate OOMW, as they did not show a significant role in the removal of organic compounds from this wastewater at the laboratory scale. The use of shells in either natural habitats or industrial facilities do not entail the same ecological and economical risks identified when using the living organism, and the industrial collection of this biogenic material is easy through aquaculture or pest management programmes. These are advantages that, together with previous evidence from the literature on the potential of bivalve shells to treat industrial effluents, should stimulate further research in this field despite our less fortunate trial. In the future, adsorption studies using Asian clam shells should test other types of organic wastewaters and metallic ions (incorporated or not in industrial effluents), in parallel to sustainable applications to the shells used in wastewaters treatments. It will also be important to investigate the performance of broken shells to fully comprehend the effect of increasing the surface area contacting with the effluent and of the higher porosity of shell's inner surfaces in the adsorption process. In this regard, we suggest an experiment focused on the use of broken shells of *C. fluminea* exposed to a more diluted fraction of OOMW.

4.5. Acknowledgments

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4.7. Supplementary material

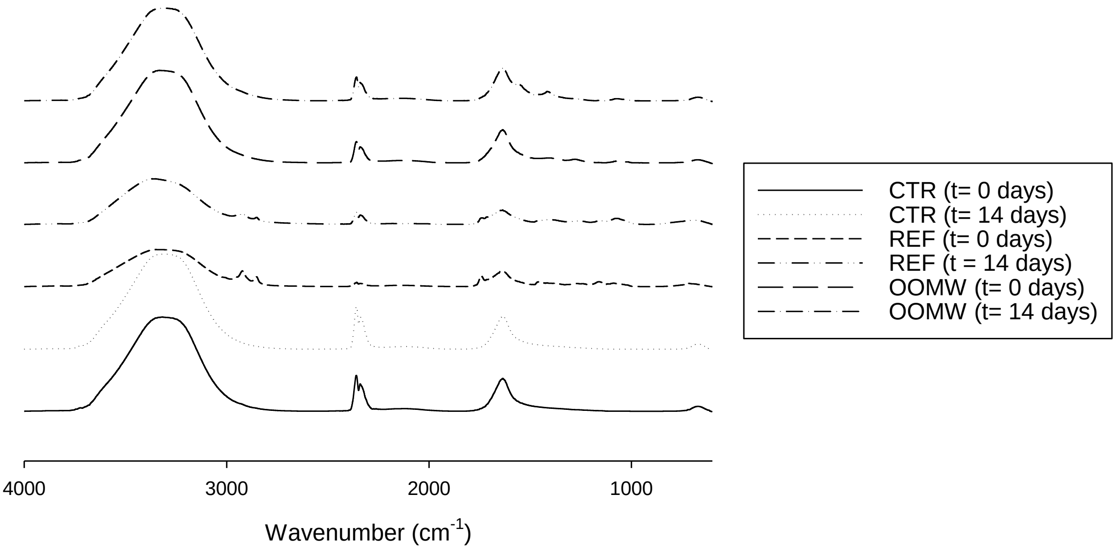

Figure S1. ATR-FTIR spectra in the region of 4000-900 cm^{-1} regarding the CTR, REF and OOMW treatments, following a 14-day filtration period. Each spectrum results from the mean of 3 replicates per treatment.

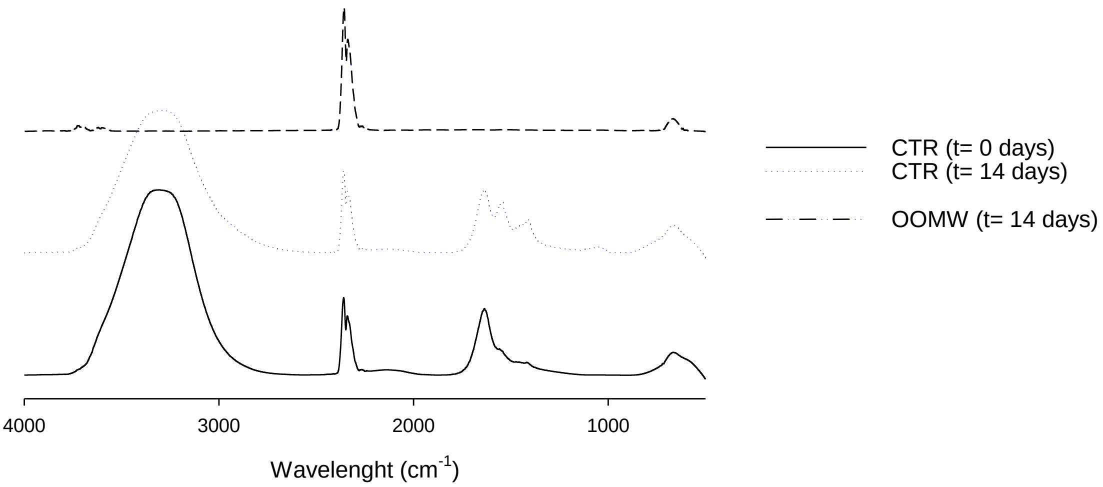

Figure S2. ATR-FTIR spectra in the region of 4000-900 cm^{-1} regarding shells of *C. fluminea* exposed to CTR and OOMW treatments, following a 14-day filtration period. Each spectrum results from the mean of 3 replicates per treatment.

Table S1 – Colorimetric results – CDOC (320 nm) and absorbances (270 and 465 nm) - registered for CTR, REF and OOMW treatments at day 0 and day 14, respectively.

Treatment		Absorbance (270 nm)	CDOC (320 nm)	Absorbance (465 nm)
CTR	Day 0	0.0120	0.310	0.00130
	Day 14	0.440	49.3	0.110
REF	Day 0	2.77	641	2.77
	Day 14	4.00	641	4.00
OOMW	Day 0	2.77	634	2.70
	Day 14	4.00	629	4.00

Table S2 – Statistical analysis (two-tailed paired-sample t tests) of CTR, REF and OOMW treatments between t= 0 days and t= 14 days.

Wavenumber (cm^{-1})	Compared Samples	Statistical Analysis (two-tailed paired-sample t tests)
3650-3000	CTR (t= 0 days) – CTR (t= 14 days)	$t_{0.05(2),2}$= -0.240 p= 0.832
	REF (t= 0 days) – REF (t= 14 days)	$t_{0.05(2),2}$= -1.05 p= 0.402
	OOMW (t= 0 days) – OOMW (t= 14 days)	$t_{0.05(2),2}$= -1.01 p= 0.417
3000-2810	CTR (t= 0 days) – CTR (t= 14 days)	$t_{0.05(2),2}$= 2.59 p= 0.122
	REF (t= 0 days) – REF (t= 14 days)	$t_{0.05(2),2}$= -0.583 p= 0.619
	OOMW (t= 0 days) – OOMW (t= 14 days)	$t_{0.05(2),2}$= -0.897 p= 0.464
1750-1180	CTR (t= 0 days) – CTR (t= 14 days)	$t_{0.05(2),2}$= 0.721 p= 0.546
	REF (t= 0 days) – REF (t= 14 days)	$t_{0.05(2),2}$= -0.838 p= 0.490
	OOMW (t= 0 days) – OOMW (t= 14 days)	$t_{0.05(2),2}$= -5.03 p= 0.0373
1180-900	CTR (t= 0 days) – CTR (t= 14 days)	$t_{0.05(2),2}$= 1.09 p= 0.389
	REF (t= 0 days) – REF (t= 14 days)	$t_{0.05(2),2}$= -0.554 p= 0.635
	OOMW (t= 0 days) – OOMW (t= 14 days)	$t_{0.05(2),2}$= 0.514 p= 0.658

Table S3 – Statistical summary of two-tailed paired-sample t tests comparing the peak areas found for shells at the beginning of the experiment (t = 0 days) and those found in shells exposed to CTR and OOMW at the end of the experiment (t = 14 days).

Wavenumber (cm^{-1})	**Compared shell samples**	**Statistical Analysis (two-tailed paired-sample t tests)**
3650-3000	CTR (t= 0 days) – CTR (t= 14 days)	$t_{0.05(2),2}$= 2.73 p= 0.223
	CTR (t= 0 days) – OOMW (t= 14 days)	$t_{0.05(2),2}$= 1.90 p= 0.198
3000-2810	CTR (t= 0 days) – CTR (t= 14 days)	$t_{0.05(2),2}$= -2.73 p= 0.112
	CTR (t= 0 days) – OOMW (t= 14 days)	$t_{0.05(2),2}$= 2.03 p= 0.179
1750-1180	CTR (t= 0 days) – CTR (t= 14 days)	$t_{0.05(2),2}$= -4.73 p= 0.0419
	CTR (t= 0 days) – OOMW (t= 14 days)	$t_{0.05(2),2}$= 2.14 p= 0.166
1180-900	CTR (t= 0 days) – CTR (t= 14 days)	$t_{0.05(2),2}$= -2.01 p= 0.182
	CTR (t= 0 days) – OOMW (t= 14 days)	$t_{0.05(2),2}$= 1,02 p= 0.416

Table S4 – Statistical summary for two-tailed simple t tests addressing the differences between variation in peak areas through the experiment in shells from the CTR and OOMW treatments.

Wavenumber (cm^{-1})	**Compared shell samples**	**Statistical Analysis (two-tailed** simple **t tests)**
3650-3000	CTR (t= 14 days) – OOMW (t= 14 days)	$t_{0.05(2),2}$= 31.2 p= $7.26*10^{-5}$
3000-2810	CTR (t= 14 days) – OOMW (t= 14 days)	$t_{0.05(2),2}$= 10.8 p= 0.000410
1750-1180	CTR (t= 14 days) – OOMW (t= 14 days)	$t_{0.05(2),2}$= 36.3 p= $3.44*10^{-6}$
1180-900	CTR (t= 14 days) – OOMW (t= 14 days)	$t_{0.05(2),2}$= 2.29 p= 0.0840

CHAPTER 5. Final Considerations

Industrial pollution continues to be a major issue in the world, one of the reasons being the anthropogenic negligence regarding the discharge of its resulting wastewaters to aquatic and terrestrial systems (Rockström et al. 2009). In the Mediterranean region, olive oil mill wastewaters (OOMW) are a major environmental concern because of its intense production (IOOC 2017), which seasonally corresponds to high volumes of these toxic industrial effluents (Gebreyohannes et al. 2016). Furthermore, the absence of a proper treatment strategy capable of reducing the dark colour and toxicity of OOMW is still a reality, and so the exploitation of novel solutions remains imperative. Biotreatments are arising as promising tools, as they present a sustainable, cost-efficient and environmentally friendly solution compared to chemical-based treatments (Paraskeva & Diamadopoulos 2006). Overall, this scenario inspired the present investigation towards the field of bioremediation.

The Asian clam *Corbicula fluminea* is a freshwater invasive bivalve, with a wide and non-controlled distribution in the Mediterranean countries, that possess a huge filter-feeding potential (Castro et al. 2018; Sousa et al. 2014; Gomes et al. 2018), which was the reason behind its hypothesised use as a bioremediation agent towards OOMW. This Dissertation presents a detailed analysis on the potential of the Asian clam towards the remediation of OOMW, as it investigates the accumulation of organic compounds in its main biological compartments: shell and soft tissues. A set of two experiments were performed in this work: a biofiltration assay, using living *C. fluminea* exposed to centrifuge wastewater effluents (CWE); and a biosorption assay that combined only the shells of the Asian clam with OOMW.

Results from the biofiltration assay immediately revealed that living organisms were capable of significantly remove organic chemicals from CWE. In parallel, both soft tissues and shells were capable of significantly accumulating chemicals as indirectly seen from changes in the molecular composition of these matrices following exposure to CWE. However, the full loss from CWE during the biotreatment did not consistently reflects in the gains monitored in the biological compartments of the clam. Although *C. fluminea* was able to filter a fraction of this highly recalcitrant wastewater, which was by itself an encouraging result, part of the filtered CWE was not accumulated but most probably concentrated in the form of pseudofaeces and then released again to the outer medium (bypass mechanism). Despite pseudofaeces represent an important factor in investigation focused on the bioremediation potential of the Asian clam, they were not analysed in this dissertation due to the huge organic complexity associated with OOMW. Still, if proven effective in the future, pseudofaeces are a path to naturally flocculate organic contaminants from OOMW, which can be an interesting avenue to explore further in the biotreatment of these wastewaters. It is worth remarking that the effectiveness of biofiltration by Asian clam was reinforced through the results of the ecotoxicological bioassays performed with CWE before and after treatment with the clams. These results showed a clear reduction in the toxicity of CWE after the biotreatment, particularly evident in the tests using the macrophyte *Lemna minor*.

As for the biosorption experiment, shells did not achieve satisfactory levels regarding the adsorption of OOMW compounds, which suggests that the accumulation in shells is only viable in living organisms. Notwithstanding, it would still be interesting to investigate the exposure of broken shells to a more diluted solution of OOMW to uncover the role of the superficial area and the increase in exposure of the inner, more porous surface of the shell to the effluent. These features could possibly improve the capacity of the shells per se within the remediation of OOMW.

In fact, despite the already positive results in the present studies, there are still several avenues that are worth pursuing in the future to fully characterise and explore the potential of living *C. fluminea* as a bioremediation agent towards OOMW. For instance, not all *C. fluminea* individuals survived during the biofiltration experiment, which means that the ideal conditions to reach the maximum filtration potential of the clam must be investigated. For example, the initial pH of OOMW is slightly acidic, which means that the clams are exposed to unfavourable conditions that could impair their normal metabolism (Mackie & Claudi 2010), thus decreasing their capacity as bioremediation agents. Thereby, a possible solution to overcome this problem is an initial pre-treatment of the wastewater like the Fenton’s process, as reported in similar studies using winery wastewaters (Pipolo et al. 2017; Ferreira et al. 2017). Physiological damage should also be studied through the biofiltration period, as they are preponderant in conditioning the filtration process and thus its efficiency rates (Marescaux et al. 2016; Way et al. 1990). In addition, the acknowledgement that a multistage assembly system should be more effective than a single-step treatment was already suggested by other authors (Rosa et al. 2014) and may apply for these effluents.

The application of invasive bivalves in industrial settings for wastewaters decontamination purposes is a possibility, but it is important to ensure the safety of such as an approach regarding the huge dispersal potential of these organisms. Also, the problem of collecting organisms could be solved through agreements towards integration with pest management programmes into place to respond to industrial biofouling, resulting in a more cost-effective solution to both industries and invaded habitats, or even other infested industries (Pipolo et al. 2017). At last, it is necessary to address the issue on the final destiny of the clams after its use for bioremediation purposes - disposal in landfills, incineration and aerobic digestion are some of the typical solutions applied to these organisms (Magni et al. 2015; Gomes et al. 2018; Pipolo et al. 2017). Overall, the use of *C. fluminea* has the potential to be a step further in the development of a suitable treatment for the problematic olive oil wastewaters and, in the meantime, also presents a sustainable and relevant management complement in pest management programmes targeted at this invasive pest.

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