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**Avaliação da eficácia do tratamento químico com  
nanomateriais na melhoria da qualidade de diferentes  
efluentes industriais**

**Assessing the effectiveness of chemical treatment  
with nanomaterials in improving the quality of  
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Dissertação apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica da Doutora Ruth Maria de Oliveira Pereira, Professora Auxiliar do Departamento de Biologia da Faculdade de Ciências da Universidade do Porto, da Doutora Isabel Maria Cunha Antunes Lopes, Investigadora auxiliar do Centro de Estudos do Ambiente e do Mar (CESAM) da Universidade de Aveiro e da Doutora Teresa Alexandra Peixoto da Rocha Santos, Professora Associada no ISEIT/Viseu do Instituto Piaget



Aos meus pais e marido pelo incansável apoio.

**“Apesar de tudo, a loucura não é assim uma coisa  
tão feia como muita gente julga. Há tantas loucas felizes!”**

**Florabela Espanca**



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## palavras-chave

efluentes industriais, nanomateriais, ecotoxicidade, organismos aquáticos, degradação fotocatalítica, tratamento biológico.

## resumo

As atividades industriais são uma das principais fontes de poluição em todos os ambientes. Dependendo do tipo de indústria, vários tipos de contaminantes orgânicos e inorgânicos são frequentemente libertados no meio ambiente, em concentrações que podem representar riscos para os meios aquáticos. Várias metodologias, nomeadamente físicas, químicas, biológicas ou a combinação de processos, têm sido propostas e aplicadas para minimizar o impacto destes efluentes, contudo nenhuma delas foi totalmente eficaz em termos de taxas de redução dos vários contaminantes, redução da toxicidade, e melhoria de algumas propriedades físicas e químicas dos diversos tipos de águas industriais residuais. É por isso imperativo o desenvolvimento de novas metodologias de tratamento, com recurso a novos materiais que vão surgindo no mercado. Neste contexto surge a nanotecnologia, oferecendo novas possibilidades no tratamento de efluentes com base nas propriedades físicas e químicas melhoradas dos nanomateriais (NMs), que podem aumentar consideravelmente o seu potencial de adsorção e oxidação. Embora as várias aplicações dos NMs possam trazer benefícios, o seu uso também irá contribuir para a sua introdução no ambiente e varias preocupações têm vindo a ser levantadas sobre estes materiais. As mesmas propriedades que tornam os NMs tão atrativos para aplicações ambientais também podem ser responsáveis por efeitos ecotoxicológicos nas comunidades naturais. Com o objetivo de selecionar NMs para o tratamento de efluentes orgânicos e inorgânicos avaliamos a toxicidade de nanopartículas de óxido de níquel (NiO) com 100 e 10-20 nm, de dióxido de titânio (TiO<sub>2</sub>, < 25 nm) e de óxido de ferro (Fe<sub>2</sub>O<sub>3</sub>, ≈ 85x425 nm). A avaliação ecotoxicológica foi realizada através de uma bateria de ensaios com organismos aquáticos de diferentes níveis tróficos e grupos funcionais. Numa segunda etapa os dois NMs pré-selecionados foram aplicados no tratamento de efluente de lagar de azeite. Os NMs foram assim usados como catalisadores em processos de fotodegradação que incluíram os seguintes sistemas TiO<sub>2</sub>/UV, Fe<sub>2</sub>O<sub>3</sub>/UV, TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub>/UV e Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub>/UV. Utilização do TiO<sub>2</sub> ou Fe<sub>2</sub>O<sub>3</sub> com H<sub>2</sub>O<sub>2</sub> revelou resultados bastante eficientes na melhoria de algumas propriedades químicas, no entanto, apesar de se ter verificado alguma redução da toxicidade para *V. fischeri*, a maior percentagem de redução foi registada para o sistema H<sub>2</sub>O<sub>2</sub>/UV, sem a adição de NMs. Posteriormente foi também aplicado ao mesmo efluente um tratamento sequencial utilizando oxidação catalítica com NMs e fungos. Esta nova abordagem aumentou a redução da carência química de oxigénio, do conteúdo em fenóis e da toxicidade para *V. fischeri*, no entanto não se verificou qualquer redução de cor e compostos aromáticos após 21 dias de tratamento biológico. Os processos de fotodegradação foram também aplicados no tratamento de efluente de pasta de papel kraft e efluente mineiro. Para o efluente orgânico a combinação NMs com H<sub>2</sub>O<sub>2</sub> resultou em eficientes reduções

quer nas propriedades químicas, quer em termos de redução da toxicidade. No entanto, para o efluente mineiro os tratamentos mais eficazes ( $\text{TiO}_2/\text{UV}$  e  $\text{Fe}_2\text{O}_3/\text{UV}$ ) apenas foram capazes de remover significativamente três metais (Zn, Al e Cd). Não obstante, estes tratamentos foram capazes de reduzir a toxicidade do efluente. Numa última etapa, avaliou-se a toxicidade dos resíduos sólidos formados após o tratamento de efluentes com NMs em larvas de *Chironomus riparius*, uma espécie representativa do compartimento sedimentos, em meios de água doce. Alguns desses resíduos mostraram a capacidade de afetar negativamente a sobrevivência e o crescimento de *C. riparius*, dependendo do efluente tratado, que os originou. Este trabalho abre novas perspectivas para a utilização de NMs no tratamento de efluentes industriais, e demonstra que apesar das aplicações anunciadas para os NMs, ainda são necessárias diversas avaliações, quer em termos da sua eficácia, quer da sua perigosidade para o ambiente antes da sua aplicação em grande escala.

## keywords

industrial wastewater, nanomaterials, ecotoxicity, aquatic organisms, photocatalytic degradation, biological treatment.

## abstract

Industrial activities are the major sources of pollution in all environments. Depending on the type of industry, various levels of organic and inorganic pollutants are being continuously discharged into the environment. Although, several kinds of physical, chemical, biological or the combination of methods have been proposed and applied to minimize the impact of industrial effluents, few have proved to be totally effective in terms of removal rates of several contaminants, toxicity reduction or amelioration of physical and chemical properties. Hence, it is imperative to develop new and innovative methodologies for industrial wastewater treatment. In this context nanotechnology arises announcing the offer of new possibilities for the treatment of wastewaters mainly based on the enhanced physical and chemical properties of nanomaterials (NMs), which can remarkably increase their adsorption and oxidation potential. Although applications of NMs may bring benefits, their widespread use will also contribute for their introduction into the environment and concerns have been raised about the intentional use of these materials. Further, the same properties that make NMs so appealing can also be responsible for producing ecotoxicological effects. In a first stage, with the objective of selecting NMs for the treatment of organic and inorganic effluents we first assessed the potential toxicity of nanoparticles of nickel oxide (NiO) with two different sizes (100 and 10-20 nm), titanium dioxide (TiO<sub>2</sub>, < 25 nm) and iron oxide (Fe<sub>2</sub>O<sub>3</sub>, ≈ 85x425 nm). The ecotoxicological assessment was performed with a battery of assays using aquatic organisms from different trophic levels. Since TiO<sub>2</sub> and Fe<sub>2</sub>O<sub>3</sub> were the NMs that presented lower risks to the aquatic systems, they were selected for the second stage of this work. Thus, the two NMs pre-selected were tested for the treatment of olive mill wastewater (OMW). They were used as catalyst in photodegradation systems (TiO<sub>2</sub>/UV, Fe<sub>2</sub>O<sub>3</sub>/UV, TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub>/UV and Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub>/UV). The treatments with TiO<sub>2</sub> or Fe<sub>2</sub>O<sub>3</sub> combined with H<sub>2</sub>O<sub>2</sub> were the most efficient in ameliorating some chemical properties of the effluent. Regarding the toxicity to *V. fischeri* the highest reduction was recorded for the H<sub>2</sub>O<sub>2</sub>/UV system, without NMs. Afterwards a sequential treatment using photocatalytic oxidation with NMs and degradation with white-rot fungi was applied to OMW. This new approach increased the reduction of chemical oxygen demand, phenolic content and ecotoxicity to *V. fischeri*. However, no reduction in color and aromatic compounds was achieved after 21 days of biological treatment. The photodegradation systems were also applied to treat the kraft pulp mill and mining effluents. For the organic effluent the combination NMs and H<sub>2</sub>O<sub>2</sub> had the best performances in reduction the chemical parameters as well in terms of toxicity reduction. However, for the mine effluent the best (TiO<sub>2</sub>/UV and Fe<sub>2</sub>O<sub>3</sub>/UV) were only able to significantly remove three metals (Zn, Al and Cd).

Nonetheless the treatments were able of reducing the toxicity of the effluent. As a final stage, the toxicity of solid wastes formed during wastewater treatment with NMs was assessed with *Chironomus riparius* larvae, a representative species of the sediment compartment. Certain solid wastes showed the potential to negatively affect *C. riparius* survival and growth, depending on the type of effluent treated. This work also brings new insights to the use of NMs for the treatment of industrial wastewaters. Although some potential applications have been announced, many evaluations have to be performed before the up-scaling of the chemical treatments with NMs.

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Table IV.4- Results of two-way ANOVA testing the effects of biological treatment period (0 and 21 days) with *P. sajor caju*, chemical pre-treatment (nano-TiO<sub>2</sub>/UV, nano-Fe<sub>2</sub>O<sub>3</sub>/UV, nano-TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub>/UV and nano-Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub>/UV) and their interaction on absorbance at 465 nm and 270 nm, pH, COD, total phenolic content and toxicity. One-way ANOVA followed by Tukey multiple comparison test ( $p < 0.05$ ) was used to evaluate statistical differences between 0 and 21 day for each pre-treatment applied.

Table IV.5- Results of two-way ANOVA testing the effects of fungi (*P. chrysosporium* and *P. sajor caju*), chemical pre-treatment (nano-TiO<sub>2</sub>/UV, nano-Fe<sub>2</sub>O<sub>3</sub>/UV, nano-TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub>/UV and nano-Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub>/UV) and their interaction on absorbance at 465 nm and 270 nm, pH, COD, total phenolic content and toxicity. One-way ANOVA followed by Tukey multiple comparison test ( $p < 0.05$ ) was used to evaluate statistical differences between fungi to each pre-treatment applied.

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Table V.3- Values of EC<sub>50</sub> recorded for bioluminescence inhibition expressed in effluent percentage, and corresponding 95% confidence intervals (CI95), or the highest effect expressed in bioluminescence inhibition percentage for OOMW samples after the treatments at 5, 15, and 30 min exposure of *V. fischeri* (HE – highest effect).

Table V.4- Values of EC<sub>50</sub> for bioluminescence inhibition expressed in effluent percentage, and corresponding 95% confidence intervals (CI95), or the highest effect expressed in bioluminescence inhibition percentage for mining effluent samples after the treatments at 5, 15, and 30 min exposure of *V. fischeri* (HE – highest effect).

Table VI.1- Mass (mg Kg<sup>-1</sup>) of the SRs containing nano-TiO<sub>2</sub> and nano-Fe<sub>2</sub>O<sub>3</sub> in the spiked sediment.



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# Chapter I

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General Introduction



## General Introduction

### 1.1 Wastewater discharges and treatment: European and national regulations

Surface and ground water resources are continuously facing profound changes and quality deterioration, caused by anthropogenic activities, such as mining operations, manufacturing and agro-industries, highly dependent from water, both for supplying processes with high quality water, but also as receiving medium of resulting wastewaters, one of the negative externalities of industries. With the industrial development required to guarantee the economic growth of countries, the generation and accumulation of waste products has tremendously increased and one of the major challenges is the proper management and safe disposal of the vast amount and array of such solid and liquid wastes. Industrial wastewaters are one of the major sources of direct and often continue input of pollutants into aquatic ecosystems (Kanu and Achi, 2011; Smolders et al., 2004). Due to the lack of effluent treatment facilities, proper treatment methodologies and disposal systems, huge amounts of industrial wastewater, containing high loads of organic and inorganic chemicals, some of them with high toxicity and recalcitrant potential, are being discharged into aquatic environments. Depending on the type of industry, the wastewater produced can contain different pollutants such as dyes, phenolic compounds, surfactants, pharmaceuticals, pesticides, organic solvents, chlorinated by-products, metals and microorganisms, which can cause the increase in biological oxygen demand (BOD), chemical oxygen demand (COD) and total dissolved solids (TDS) in the water systems promoting their deterioration (Kanu and Achi, 2011; Muñoz et al., 2009; Weber et al., 2006). This environmental contamination is now a global concern and managing these wastewaters is becoming increasingly crucial for ensuring a clean environment, in light of significant water scarcity being faced by many countries. The United Nations (U.N.) Millennium Ecosystem Assessment (MA) highlighted the need for the preservation of aquatic resources for both ecosystem integrity and human well being. Ecosystem services are the benefits that people obtain from ecosystems however, despite their critical importance, the MA reported that over 60 percent of ecosystem services were

already degraded and will continue to be degraded for decades to come (MA, 2005). Wetlands deliver myriad ecosystem services that contribute to human well being, being fish supply and water availability the two most important services affecting human well-being. Thus, unfortunately the degradation and loss of these ecosystems is more rapid than that of other ecosystems. Population growth and increasing economic development are the primary indirect causes of degradation and loss of wetland ecosystems, but also direct causes like pollution, water withdrawal, overharvesting and overexploitation, and climate change are meaningful (MA, 2005). Hence, at the European level, strict environmental legislation like the Water Framework Directive (WFD, 2000/60/EC) and corresponding daughter directives (e.g., Directive 2008/1/EC, 91/271/EC and 2008/105/EC) (EC, 2000, 2008a,b) aimed in protecting these resources came into force, with a new ecosystemic vision of the freshwater ecosystem as well as with a more conservative approach regarding wastewater discharges. This new approach was particularly interesting since most of the contaminants found in wastewaters can easily find their way to groundwater and surface waters resources being responsible for affecting their chemical and ecological conditions (Fatta-Kassinos et al., 2011; Kanu and Achi, 2011). In 2005 the Portuguese Water Law (Law nº 58/2005, of 29 of December) transposed the EU WFD (AR, 2005).

The article 1 of the decision nº 2455/2001/EC (EC, 2001) of the European Parliament and of the Council of 20 November of 2001 established a first list of 33 priority substances including priority hazardous substances that pose a significant risk to the aquatic environment (EC, 2001). Priority and hazardous substances were defined by the WFD as *“substances that represent a meaningful risk to aquatic environment, which should be identified by normal risk assessment procedures”* and, *“as substances or groups of substances that are toxic, persistent and liable to bioaccumulate, and other substances or groups of substances which give rise to an equivalent level of concern”*, respectively. Within these substances were metals like cadmium, lead and mercury, phenolic compounds (nonylphenols, octylphenols and pentachlorophenols) as well dioxins. This decision amended the WFD and the list was added to Directive 2000/60/EC as Annex X, with the main aim of controlling emissions,

discharges and losses of priority substances and of phasing out emissions, discharges and losses of priority hazardous substances (article 1).

The WFD daughter Directive 2008/105/EC, of 16 of December 2008, on environmental quality standards in the field of water policy laid down the environmental quality standards for the priority substances (EQS) and other pollutants included in the annex (EC, 2008a). This Directive was transposed to the national legislation by the Law by Decree nº 103/2010 of 24 of September which included in its Annex I the list of priority substances and in annex II, the list of other pollutants, while annex III set the EQS for surface waters (MAOT, 2010). Thus, in order to regulate the discharge of these substances and achieve good surface water chemical status, water bodies must meet environmental quality standards (EQS) set by the Directive 2008/105/EC (EC, 2008a).

The WFD also expected that member states establish an inventory of emissions, discharges and losses of the all priority substances and other pollutants listed in Part A of Annex I of the Directive 2008/105/EC for each river basin district, but also, for some of these compounds, for the sediment and biota (EC, 2008a). The great majority of chemical substances are not included in the previous list for which EQS were defined and, that must be monitored, according to the above mentioned legislation, but they can be detected, at least indirectly, if they harm the ecological communities of receptors systems. In this context the WFD is complemented with two directives addressing wastewater discharges into surface water, the Urban Wastewater Treatment Directive (UWTD, Directive 91/271/EEC) and the Industrial Emissions Directive (IED - Directive 2010/75/EU) (EC, 1991; EC, 2010). The IED groups seven directives, including the Integrated Pollution Prevention and Control Directive (IPPC, Directive 2008/1/EC), into one piece of legislation. The IPPC directive applies to large industries with a high pollution potential such as the energy sector, the production and processing of metals, the mineral and chemical industries, pulp and paper production, waste management facilities, food production and non-industrial activities such as livestock farming, as defined in Annex I of the Directive. This directive introduced an integrated and combined approach intended to control the emissions to air, water and

soil, applying the best available techniques (BAT) in order to ensure a high level of protection of the environment taken as a whole. Also it established emission limit values (ELVs) for a list of polluting substances (e.g. metals and their compounds, persistent hydrocarbons, substances which have an unfavourable influence on the oxygen balance, as well the substances listed in Annex X to Directive 2000/60/EC) defined in Annex III of the Directive based on BAT, controlling simultaneously point-source pollution and ensuring the compliance with the applicable environmental quality standards (EC, 2008b). IPPC enforces the treatment of wastewaters prior to discharge by applying preventive and minimization techniques. The Law by Decree nº 127/2013, of 30 of August, transposed into national law the Industrial Emissions Directive applicable to the prevention and control of pollution, as well as rules to prevent and/or reduce emissions to air, water and soil, and waste generation in order to achieve a high level of environmental protection as a whole (MAOTE, 2013). The Portuguese legislation also established in the Annex XVIII of the Law by Decree 236/98 of 1 August, emission limit values (ELVs) for the discharge of industrial wastewaters (ME, 1998). To attain a good chemical and ecological status of water masses, as required by the WFD and the Portuguese legislation, is necessary to properly treat industrial wastewaters before their discharge to aquatic ecosystem to reduce their environmental impact. Recognizing the clear evidence that our water resources are increasingly scarce in various regions and countries, and that the quality of the available freshwater is also deteriorating due to pollution, the research for new wastewater treatment methodologies are increasing worldwide.

## **1.2 Some of the most concerning wastewaters**

### **1.2.1 Olive oil mill wastewaters (OOMW)**

Olive oil production is one of the most important agricultural industries in the Mediterranean basin countries. According to the International Olive Oil Council from November 2013, 2.4 millions of tones of olive oil are produced annually worldwide being the major producers in the EU (72.1%), namely in Spain (67.4.0%), Italy (16.7%), Greece (12.3%) and Portugal (3.2%). In the rest of the world, yet in the Mediterranean



area, Tunisia (5.5%), Syria (6.0%), Turkey (5.8%) and Morocco (3.6%), are relevant olive oil producers. However, outside Mediterranean basin, other countries are also becoming emergent producers like Argentina, Australia and South Africa (Paraskeva and Diamadopoulos, 2006; Roig et al., 2006). Regardless of the economic advantages, the olive oil has also been associated with health benefits, such as lower incidence of cardiovascular disease and certain types of cancer (prostate and colon). The occurrence of such health problems in Greece, Italy and Spain are considerably lower and this has been related with the Mediterranean diet, which includes olive oil as a relevant food item (Cicerale et al., 2010; Tuck and Hayball, 2002; Visioli and Galli, 1998).

However, the extraction of olive oil poses two serious environmental problems for the producing countries; on the one hand large amounts of water are used in the extraction process and in the other huge quantities of high extremely toxic wastewaters and sludge are generated (Arvaniti et al., 2012; Dermeche et al., 2013). More than 30 million m<sup>3</sup> of wastewater are generated every year in the world (Kavvadias et al., 2010; McNamara et al., 2008).

Nowadays, the traditional process (pressing) and centrifugation technologies such as three- and two-phase centrifugation systems are the two ways of extracting the oil. Although the pressing process is a relatively obsolete technology and its use has decreased, it is still being used for some small olive oil mills especially due to the cheap equipment and technical simplicity of the processes carried out. However, it produces a more concentrated OOMW compared to the centrifugation systems, because uses a small quantity of water (Bhatnagar et al., 2014; Dermeche et al., 2013). The three-phase method produces olive oil, a solid residue (olive pomace), and a great amount of OOMW, because of the water that is added at different steps during oil production to improve the separation of oil (Peikert et al., 2014). This led to the introduction in the early nineties of a two-phase “ecological friendly” extraction system. This process produces two products: olive oil and a by-product waste, which is the combination of olive husk and OOMW (called wet pomace or two-phase olive mill waste (TPOMW)) (Dermeche et al., 2013; Roig et al., 2006; Tsagaraki et al., 2007). The

main advantage of this system is the significant reduction in the volume of OOMW produced, nevertheless large amounts of semi-solid waste (TPOMW) are still produced (Albuquerque et al., 2004; Dermeche et al., 2013; Justino et al., 2012; Morillo et al., 2009).

In summary, the management of wastes from olive oil extraction has to deal with three main problems: the seasonality of waste generation, the enormous volume of wastewater generated each year, and the other types of waste. The complex physical and chemical composition of olive oil mill wastewater (OOMW) is variable and depends on many factors such as olive variety and maturity, climatic conditions, the local and seasonal nature of oil production, cultivation methods and the extraction process (McNamara et al., 2008; Ouzounidou et al., 2010; Roig et al., 2006). Nevertheless, the main composition of olive oil mill wastewater is about 83-96% of water, 1-8% of sugars, 0.5-2.4% of nitrogenous compounds, 0.5-1.5% of organic acids, 1.0-1.5% of phenols, pectin and tannins, 0.02-1.0% of lipids and inorganic substances, with offensive smell and dark colour (Asses et al., 2009; Ouzounidou et al., 2010; Sayadi et al., 2000). The main environmental impacts of OOMW derive from its acidic condition (pH 3.0-5.9), high organic load (biological and chemical oxygen demands can reach up to 100 and 220 g L<sup>-1</sup>, respectively) and phenolic content (up to 80 g L<sup>-1</sup>) (Azbar et al., 2004), which contribute for the high toxicity of this wastewater. It is generally acknowledged that the high toxicity of OOMW is due to polyphenolic components since these compounds are highly recalcitrant and toxic to microorganisms and plants (Asfi et al., 2012; Saadi et al., 2007).

Despite the recognition that OOMW is a truly hazard residue, in many countries it is still being spread onto soil or into water courses, without any valorisation treatment, causing negative impacts on the environment (Peikert et al., 2014). For example, S'habou et al. (2009) shows that the uncontrolled and repeated disposal of OOMW can affect soil quality, increasing the organic matter, the electrical conductivity and phenolic compounds concentration, as well contaminate groundwater with phenolic compounds. However, and despite the known hazard of this wastewater, the Portuguese Ministry of Agriculture, Rural Development and Fishing, aimed in reducing

the uncontrolled disposal and in promoting some valorisation to this waste, gave a special permission through the regulatory order Nº 626/2000, for spreading OOMW on land (MADRP, 2000). Thus a limit of discharge in soils of  $< 80 \text{ m}^3/\text{ha}/\text{year}$  for irrigation purposes was established. And this use can be made close to water bodies. Nevertheless, the most common method of elimination in practice has been the storage in lagoons where OOMW is left to evaporate naturally, until the production of the next year. Despite being the most economical method such disposal only reduces the volume of wastewater concentrating the most hazardous pollutants, causing several environmental problems such as bad odour or the possible contamination of soil and water body pollution (de la Casa et al., 2009; Kavvadias et al., 2010; S'habou et al., 2009). Thereby, several other treatments options have been proposed and tested, such as physicochemical (membrane filtration, coagulation-flocculation, electrocoagulation, advanced oxidation, ultra-filtration/reverse osmosis, adsorption and sedimentation) and biological (aerobic or anaerobic processes) as well several combined technologies (for instance a sequential treatment with fungi and photo-Fenton), aiming to make OOMW acceptable for discharge into the environment (Achak et al., 2014; Arvaniti et al., 2012; Duarte et al., 2014; Hanafi et al., 2010; Hodaifa et al., 2013; Jarboui et al., 2013; Kilic and Solmaz, 2013; Michael et al., 2014; Pelendridou et al., 2013; Sampaio et al., 2011). Although some treatment options have obtained significant results in ameliorating parameters as colour, COD and phenolic content the toxicity persisted or in some cases found a slightly increased (Justino et al., 2012). None of these single technologies or the combination between them was effective enough to gather a consensus to be universally adopted for OOMW treatment.

### **1.2.2. Paper mill wastewaters**

The pulp and paper industry has always had to deal with two major environmental problems; the first is consumption of high quantities of freshwater and the second is the large volume of pollutant wastewater produced (Lucas et al., 2012). Although several efforts were taken in the last few years to recycle and reduce the consumption of water with the best available techniques (Gönder et al., 2011), it still

uses a considerable amount of freshwater resources, which can attain 60 m<sup>3</sup> per tonne of paper produced (Pokhrel and Viraraghavan, 2004; Thompson et al., 2001). Pulp and paper industry is one of the largest contributors to air and water pollution (Garg and Tripathi, 2011). The most significant sources of pollution in pulp and paper industry are wood preparation, pulping, pulp washing, bleaching and coating operations. Among the various processing steps, pulping is one of the major sources of pollutants in all the process because large amount of water are necessary reappearing in the form of an effluent. In this step, wood chips are digested so the fibers can be separated and treated to remove lignin. Two major processes are been used worldwide, mechanical and chemical processes, but they can also be combined depending of the desire quality of the finish product. Both in European Union and North America the most prevalent process is the chemical pulping (Badar and Farooqi, 2012; Pokhrel and Viraraghavan, 2004). In the chemical pulping high amounts of chemicals are used as alkaline (Kraft process) or acidic (sulphite process) mediums to cook and digest the wood chips in the presence of elevated pressures and temperatures. Using chemical pulp to produce paper is more expensive and the yield is lower than using mechanical pulp, but it has better strength and brightness properties. The wastewaters generated from the pulping process include solid wastes, like wood debris and several wooden compounds such as lignin, carbohydrate and wood extractives. Also, depending on the type of pulping process, resins acids, unsaturated fatty acids, diterpene alcohols, chlorinated resin acids can appear (Pokhrel and Viraraghavan, 2004). Some of the wood extractives, although in smaller quantities, can be more toxic than phenols and can decrease the quality of the aquatic environment, the final receptor of these wastes (Garg and Tripathi, 2011). The chemical pulp bleaching is the last step and the responsible for most of the toxicity of the wastewater, as in this process the objective is to remove the remained lignin and brighten the pulp. Several chemicals are used to transform lignin as oxygen, hydrogen peroxide, sodium hypochlorite, chlorine dioxide and chlorine. The effluent generated from the bleaching contains dissolved lignin, chlorinated organic compounds (AOX) as well inorganic chloride compounds (e.g., dioxins, furans and chlorophenols) (Badar and Farooqi, 2012; Covinich, 2014). Several

AOX are xenobiotics and with recalcitrant nature and tend to persist in the environment (Garg and Tripathi, 2011). Moreover, the chemical composition of pulp and paper mill effluents vary in a wide range, depending on the type of wood used and the pulping and bleaching procedures, but is characterized by high chemical oxygen demand, low biodegradability and dark-brown colour (Buyukkamaci and Koken, 2010; Wang et al., 2011). The dark-brown colour resulted from the lignin compounds present in the effluent, and are both visually unacceptable and responsible by reducing light transmission and thereby the phytoplankton activity (Garg and Tripathi, 2011; Sahoo and Gupta, 2005). The most common treatments applied to the effluents from the pulp and paper industry are the biological systems, like sequential anaerobic and aerobic treatments, after a primary clarification. However, the biological treatment is not able to completely remove all the toxic compounds, such as inorganic compounds, and a tertiary treatment (e.g. sedimentation/floatation, coagulation and precipitation, adsorption, oxidation and membrane filtration) is often needed (Gönder et al., 2011; Kamali and Khodaparast, 2014).

### **1.2.3 Mining effluents**

One of the major anthropogenic sources of metal contamination in soils and waters are the mineral and mining activities (Johnson and Hallberg, 2005). Over the years mines were extensively exploited been an important economic activity in many countries however, even after the cessation of the exploratory activity, the impacts persists (Antunes et al., 2007; Lopes et al., 2000; Pereira et al., 2004). There are several methods of metal and industrial minerals mining (e.g., underground mining, open-pit mining, strip mining and dredge mining) being very difficult to predict the composition of the wastewater produced (Cooke and Johnson, 2002; Iakovleva and Sillanpää, 2013). Although the extraction method and the composition of the base minerals in the rock can vary, they all share a common characteristic, which is the production of acidic effluents, the so called acid mine drainage (AMD) (Cooke and Johnson, 2002; Williams, 2001). AMD results from the oxidation of sulphide minerals (like pyrite) when they are exposed to oxygen, water and microorganisms. When sulphur compounds are

exposed they oxidize to form acidic sulphate-rich drainage. Iron sulphides are the most common minerals produced and take pyrite ( $\text{FeS}_2$ ) as an example we can illustrate the reaction of acid generation (Johnson and Hallberg, 2005):



The AMD is characterized typically by a high acidity (pH values as low as 2), high concentration of metals and metalloids (e.g., Fe, Cu, Zn, Co, Cr, Pb, As etc.) and anions like sulphates (Cheng et al., 2011; Feng et al., 2004; Mohan and Chander, 2006). The lower values of pH promote the dissolution and release of metals transporting them to a receiving water body and causing deleteriously effects in the local biota (Hallberg, 2010; Sarmiento et al., 2011). In addition to the toxic effects due to metals, also the pH has a detrimental effect on the organisms (Tiwary, 2001). Mining drainage alters significantly the environment both esthetical as the structure and function of the habitats. Therefore, even after the mining activities have been suspended, it is a priority to establish effective methods for the treatment for these effluents. There are two main approaches in the remediation of AMD, passive and active treatments. The active treatments normally involve the addition of chemicals to increase the pH and precipitate de metals. While in the passive treatments no chemicals are added and natural processes or biological treatments are normally used (e.g. settlement lagoons or aerobic wetlands) to attain the same results of active treatment (Cheng et al., 2011; Trumm, 2010).

### **1.3. Treatments for organic and inorganic effluents**

Nowadays, faced with more stringent regulations, the search for more effective techniques for wastewater treatment increased, and although many techniques can be employed for the treatment of effluents, the ideal treatment should be not only suitable, appropriate and applicable to the local conditions and source of wastewater, but also able to meet the maximum contaminant level standards established. Conventional treatments processes, or primary water treatment technologies, such as flotation, coagulation and flocculation, membrane filtration and adsorption can remove both organic and inorganic pollutants (Gupta et al., 2012; Spellman, 2013).

In the flotation method the suspended particles attach to dissolved air or gas, forming agglomerates with lower density and causing the flock to rise to the surface of the suspension where it can easily be recovered (Gupta et al., 2012). Ion flotation (using a cationic surfactant as a collector) is a promising method for the removal of metals from wastewaters while the dissolved air flotation shown to be an effective method for treating pulp and paper mill effluent. Wenta and Hartmen (2002) showed the efficiency of flotation in removing 95% of the total suspended solids in a pulp and paper effluent (Pokhrel and Viraraghavan, 2004). Salmani et al. (2013) investigated the potential of ion flotation to remove cadmium using sodium dodecylsulfate (SDS) as collector and ethanol as frother. Metal removal reached 82.5% when the ratio of SDS to metal was 3:1 at pH=4.

The coagulation and flocculation treatment is also employed to remove metals, normally followed by sedimentation or filtration. Chang and Wang (2007) proved the efficiency of using a new kind of coagulant, sodium xanthogenate group grafted to polyethyleneimine (PEX). The combining use of the chelating and coagulating proved its ability in removing the turbidity but also metal ions (Chang and Wang, 2007). However when coagulation-flocculation was used to treat paper pulp and paper mill wastewater no reduction in COD and absorbance was recorded, although several impurities were efficiently eliminated (Rodrigues et al., 2008). Coagulation/flocculation has also been applied as a pre-treatment step in the treatment of OOMW mainly to reduce the solid fraction of this wastewater and improve the efficiency of the subsequent treatments. Several reports showed the efficiency of coagulation/flocculation processes in the reduction of TSS (total suspended solids), however the removal of COD was not so successfully achieved (Aktas et al., 2001; Ginos et al., 2006; Rizzo et al., 2008; Sarika et al., 2005).

Membrane processes such as microfiltration, ultrafiltration, nanofiltration and reverse osmosis, have received significant attention since it can be employed not only for metal's removal from inorganic effluents, but also to remove organic compounds and suspend solids, with reduced costs of installation and operation (Barakat, 2011; Coskun et al., 2010; Fu and Wang, 2011). Coskun et al. (2010) reported that 79.2% and

96.3% removal of COD was achieved after the treatment of OOMW by nanofiltration membranes and reverse osmosis membranes, respectively. While Landaburu-Aguirre et al. (2012) employed micellar-enhanced ultrafiltration to remove metals from a phosphorous rich drainage of a fertilizer company. At optimum conditions (pH 3.2 and sodium dodecyl sulphate concentration of 75.6 mM) they achieved removal rates of 84.3% and 75.0% for cadmium and copper, respectively, at optimum conditions

Another interesting treatment is adsorption and in the last years, the search for low-cost adsorbents has intensively increased (Barakat, 2011). Agricultural wastes, industrial by-products or natural material have been used as low-cost adsorbents. In a recent paper, Mohan et al. (2014) reviewed the adsorption of several organic (e.g., dyes, phenols, aromatics and solvents) and inorganics (e.g.,  $\text{Cd}^{2+}$ ,  $\text{Pb}^{2+}$ ,  $\text{As}^{3+}$  and  $\text{Cr}^{6+}$ ) by different biochars (e.g., oak wood, dried olive pomace, cow manure, pinewood, etc.). In another study, Achak et al. (2009) showed the potential of application of banana peel as adsorbent for removing phenolic compounds from a real olive mill wastewater. By increasing the dosage of banana peel, at pHs above neutrality significantly increased the phenolic compounds adsorption (Achak et al., 2009).

Another treatment widely used to remove metals from inorganic effluents is chemical precipitation, using lime and limestone for example. Unfortunately, like the methods presented earlier, chemical precipitation leads to the production of another kind of waste, such as sludge, being only a phase transfer of pollutants. The sludge production requires further treatment and disposal to prevent long-term environmental impacts (Padmanabhan et al., 2006). Owing to this, methods like chemical and biological process are preferred, able of efficient destructions of pollutants in water.

Advanced oxidation processes (AOPs) are considered as promising and effective alternatives to deal with persistent and recalcitrant pollutants or to convert pollutants that can be further treated by biological methods (Machulek Jr. et al., 2013; Oller et al., 2011). These processes (e.g., Fenton process, photocatalytic oxidation, ozonation or cavitation) generate very reactive free radicals, principally hydroxyl radicals ( $\text{HO}\cdot$ ), which are highly reactive and non-selective, and precursors of degradation of organic



and inorganic compounds (Comninellis et al., 2008; Gogate and Pandit, 2004; Machulek Jr. et al., 2013; Pera-Titus et al., 2004; Pignatello et al., 2006). Gernjak et al. (2004) showed the efficiency of solar photo-Fenton process that successfully removed up to 85% COD and up to 100% of phenol content of OOMW. In another study, El Hajjouji et al. (2008) applied photocatalysis using  $\text{TiO}_2$  to treat OOMW recording removals of 94% for phenolic compounds. Also AOPs have being used to oxidize metals to a less toxic form, for example, Dutta et al. (2005) observed the removal of arsenic through oxidation of As(III) to As(V) followed by adsorption of As(V) onto  $\text{TiO}_2$  surfaces reaching values below the World Health Organization drinking water limit.

Biological treatments are the most cost-effective, feasible and environmental well-suited processes for the removal or reduction of organic and inorganic compounds from industrial wastewater (Justino et al., 2012; Mantzavinos and Kalogerakis, 2005). The elimination of pollution is made by the metabolic activity of living organisms, frequently microorganisms, which can be fungi such as *Pleurotus sajor caju*, *Trametes versicolor*, *Phanerochaete chrysosporium*, *Pleurotus ostreatus* and *Rhizopus oryzae* (Fountoulakis et al., 2002; Rocha-Santos et al., 2010; Yetis et al., 1998); bacteria such as *Bacillus laterosporus*, *Bacillus licheniformis*, *Pseudomonas putida* and *Ralstonia sp.* (Di Gioia et al., 2001; Zouboulis et al., 2004); and yeasts such as *Yarrowia lipolytica* (Lanciotti et al., 2005). However, the utilization of biological treatment has some drawbacks and may be limited due to the presence of toxic or recalcitrant compounds (Justino et al., 2012).

#### **1.4. Nanomaterials for remediation/wastewater treatment**

In the last few years nanomaterials (NMs) with their unique proprieties have been extensively studied for water and wastewater treatment. Nanotechnology holds the promise of enhancing the performance of the existing treatment technologies but also offers the potential to develop new treatments (Qu et al., 2013). In 2011 the European Commission has adopted the following definition for nanomaterials: “a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in

*the number size distribution, one or more external dimensions is in the size range 1 nm–100 nm*” (EC, 2011). Due to its reduced size NMs display unique physical, chemical and biological proprieties compared to their bulk counterparts (Stone et al., 2010; Zhang and Fang, 2010). At nanoscale level, the specific surface area and surface volume ratio increases, leading to an increase in the number of surface atoms, therefore they present new optical, electrical and magnetic proprieties (Mohmood et al., 2013). The characteristics which make NMs suitable for applications like wastewater treatment include high surface area with more active sites available for adsorption; high reactivity and catalytic potential for use in photocatalysis; antimicrobial proprieties; high mobility in solution, as well superparamagnetism proprieties for particle separation (Hariharan, 2006; Qu et al., 2013, 2012; Sánchez et al., 2011).

These unique proprieties can be employed to remove metals or degrade persistent organic compounds. The ability of NMs to trap metals is well known, Recillas et al. (2011) explored the ability of  $\text{CeO}_2$ ,  $\text{Fe}_3\text{O}_4$  and  $\text{TiO}_2$  NPs for the removal of  $\text{Pb(II)}$  via adsorption reporting adsorption capacities of 189 mg  $\text{Pb/g}$ , 83 mg  $\text{Pb/g}$  and 159 mg  $\text{Pb/g}$ , respectively. Pena et al. (2005) reported another example, in which nanocrystalline  $\text{TiO}_2$  were used both as adsorbent to remove arsenate [ $\text{As(V)}$ ] and arsenite [ $\text{As(III)}$ ] as well to complete convert  $\text{As(III)}$  to  $\text{As(V)}$  through photocatalytic oxidation. In a recent review, Hua et al. (2012), reported several studies demonstrating the efficiency of nanosized metal oxides for metals removal, as well other strategies involving the functionalization of NMs like surface modification with amino groups, supporting with zeolites or coating with poly(3,4-ethylenedioxythiophene) to enhance the efficiency of metal removal.

Furthermore, research on the removal of organic compounds has also being performed. Iron oxide NPs found application for the efficient removal of organic pollutants (Iram et al., 2010; Parham et al., 2012; Wang and Huang, 2011; Zhang et al., 2010; Zhao et al., 2010). Special attention is being focused on the use of NMs for photocatalysis to degrade a variety of organic compounds such as dyes (Faisal et al., 2007; Giwa et al., 2012; Shu et al., 2009), phenols (Chiou et al., 2008; Morales-Flores et

al., 2011; Zhang et al., 2008), pesticides (Mahmoodi et al., 2007), drugs (El-Kemary et al., 2010) as well chlorinated aromatic compounds (Lu et al., 2011; Selli et al., 2008). The major drawbacks in these papers are typically the use of synthetic water that obviously not represent the real wastewaters, also several of this studies focus on individual compounds forgetting about the complex mixtures where they are included like industrial wastewaters.

In this context, despite all the potential benefits that can accrue from the use of NMs in water and wastewater treatment, another question arises whether the use of NMs presents additional risks in biological environments. The unique proprieties of NMs, which make them so appealing, can also be responsible for ecotoxicological effects. Hund-Rinke and Simon (2006) for example, were one of the first authors reporting the effects caused by the potential formation of ROS during UV-irradiation of TiO<sub>2</sub> nanoparticles used as photocatalysts on *Daphnia magna* and *Desmodesmus subspicatus*. Also, the nanosize can favour the cross through cell membranes and to interact with cellular components (Colvin, 2003; Li et al., 2002). There are some reviews reporting the toxicity of NMs to several organisms (Baun et al., 2008; Menard et al., 2011; Navarro et al., 2008; Peralta-Videa et al., 2011). However, there still exists a considerable gap between the available data on toxicity evaluation and the large amount of NMs that are being produced worldwide. Another factor that needs to be accessed is that in the environment the toxic effects will not only depend on morphologic proprieties, composition, size or synthesis method of NMs, but also in the physico-chemical characteristics of the medium surrounding (Hu et al., 2009; Lowry et al., 2012). Is therefore imperative access the ecotoxicity of NMs before their use for water and wastewater treatments purposes.

### **1.5. Goals and thesis structure**

Water resources are facing profound changes due to the spread of a wide range of contaminants and it is recognized that the protection and conservation of natural water resources is crucial. Effluents are a main source of direct and continuous input of pollutants in aquatic ecosystems. High loads of organic and inorganic compounds,

some of them with high toxicity and recalcitrant potential, persist in wastewater, causing serious environmental problems.

To overcome these problems, it is imperative to properly treat these wastes, with treatments capable of resolve or at least ameliorate the quality of the treated effluent to acceptable levels before discharge. Although numerous approaches have been applied (physical, chemical and biological treatments) the high variety of contaminants in wastewater turns the treatment of these wastes a difficult and expensive task without satisfactory solution. Even some promising methodologies like advanced oxidation processes which revealed great effectiveness in terms of oxidation of organic compounds, reduced colour, BOD and COD, and metal contents, little effects have in terms of reducing toxicity and in some cases even increase it. Take into consideration this assumption, the main goal of the present thesis is to evaluate the effectiveness of different NMs in improving the physicochemical proprieties of different industrial wastewaters and reducing its toxicity to the organisms of the receiving ecosystems. Thus, the present thesis is structured into seven chapters, the first and seventh chapter concern the general introduction and, conclusions and final remarks of the thesis, respectively, while the other five are related with research work, already submitted to peer reviewed journals or in preparation. Below is a brief description of the objectives for each chapter.

**[Chapter II]** The objective of this chapter was to evaluate the toxicity of four nanomaterials (NiO with two different particle sizes, Fe<sub>2</sub>O<sub>3</sub> and TiO<sub>2</sub>) with potential for wastewater treatment, by performing a battery of tests with aquatic species from different trophic levels and functional groups. This assessment was made with the bacteria *Vibrio fischeri*, the microalgae *Raphidocelis subcapitata*, the macrophyte *Lemna minor*, the cladoceran *Daphnia magna* and two marine species the rotifer *Brachionus plicatilis* and the crustacean *Artemia salina*. A screening test to access the mutagenicity of these NMs with two *Salmonella typhimurium* strains was also performed. Suspensions of each NM, prepared on the different test mediums and at different concentrations tested, were characterized by light scattering. This chapter

provided crucial knowledge to select the NM with lower risks to the environment, before assessing their potential use in the treatment of complex organic and inorganic effluents produced by industrial activities.

**[Chapter III]** The main goal of this chapter was to investigate the treatability of real olive mill wastewater true several systems involving advanced oxidation processes (AOPs) and using as catalysts two nanomaterials ( $\text{TiO}_2$  and  $\text{Fe}_2\text{O}_3$ ). Photodegradation process was compared using  $\text{H}_2\text{O}_2/\text{UV}$ , nano- $\text{TiO}_2/\text{UV}$ , nano- $\text{Fe}_2\text{O}_3/\text{UV}$ , nano- $\text{TiO}_2/\text{H}_2\text{O}_2/\text{UV}$  and nano- $\text{Fe}_2\text{O}_3/\text{H}_2\text{O}_2/\text{UV}$  systems. The effects of catalyst dose and oxidant concentration were also investigated. The efficiency of the treatment was assessed through aromatics (270 nm) and colour reduction (465 nm), chemical oxygen demand (COD), phenolic content and toxicity to *Vibrio fischeri*.

**[Chapter IV]** The objective of this chapter was to investigate the treatability of olive mill wastewater however using an approach different from the one described in chapter III. A slightly diluted OOMW (50%) was treated with the combination of photocatalytic (nano- $\text{TiO}_2/\text{UV}$ , nano- $\text{Fe}_2\text{O}_3/\text{UV}$ , nano- $\text{TiO}_2/\text{H}_2\text{O}_2/\text{UV}$  and nano- $\text{Fe}_2\text{O}_3/\text{H}_2\text{O}_2/\text{UV}$ ) and fungus (*Phanerochaete chrysosporium* and *Pleurotus sajor caju*) treatments. The efficiency of the combination of treatments was also assessed through aromatics (270 nm) and colour reduction (465 nm), chemical oxygen demand (COD), total phenolic content and ecotoxicity (Microtox®)

**[Chapter V]** In this chapter, the photocatalytic process with  $\text{TiO}_2$  and  $\text{Fe}_2\text{O}_3$  NMs was applied to treat two real hazardous wastewaters, the Kraft pulp mill effluent and mining effluent. The approach used in this chapter was similar to chapter III with the olive oil mill wastewater. The efficiency of combination of oxidant and nano-catalyst, as well nano-catalyst alone was assessed for both wastewaters. For the Kraft pulp mill we evaluated the toxicity to *V. fischeri*, aromatic compounds, colour and COD removal. While for the mining effluent the total concentration of seven metals and mettalooids (S, Cu, Zn, Al, As, Pb, Cd) and ecotoxicity before and after the treatment was assessed.

**[Chapter VI]** Finally, and due to concerns about the potential effect of nano-rich residuals produced during wastewater treatment with NMs, and which can be non-intentionally released to the environment, the main purpose of this chapter was to evaluate the solid wastes coming from the best treatments of the three effluents [olive oil mill (chapter III), kraft pulp mill and mining drainage (chapter V)]. The invertebrate *Chironomus riparius* was selected as test organism and exposed to sediments spiked with the sludge. The effect on percentage of survival and growth was assessed.

## **1.6 References**

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# Chapter II

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**Assessing the ecotoxicity of metal nano-oxides with potential for  
wastewater treatment**



## Assessing the ecotoxicity of metal nano-oxides with potential for wastewater treatment

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### Abstract

The rapid development of nanotechnology and the increasing use of nanomaterials (NMs) raise concern about their fate and potential effects in the environment, especially for those that could be used for remediation purposes and that will be intentionally released to the environment. Despite the remarkable emerging literature addressing the biological effects of NMs to aquatic organisms, the existing information is still scarce and contradictory. Therefore, aimed at selecting NMs for the treatment of organic and inorganic effluents, we assessed the potential toxicity of NiO (100 and 10-20 nm), Fe<sub>2</sub>O<sub>3</sub> (~85x425 nm) and TiO<sub>2</sub> (< 25 nm), to a battery of aquatic organisms: *Vibrio fischeri*, *Raphidocelis subcapitata*, *Lemna minor*, *Daphnia magna*, *Brachionus plicatilis* and *Artemia salina*. Also a mutagenic test was performed with two *Salmonella typhimurium* strains. Suspensions of each NM, prepared with the different test media, were characterized by Dynamic Light Scattering (DLS) and Eletrophoretic Light Scattering (ELS). For the assays with marine species no toxicity was observed for all the compounds. In opposite, statistically significant effects were produced on all freshwater species, being *D. magna* the most sensitive organism. Based on the results of this study, the tested NMs can be classified in a decreasing order of toxicity NiO(100nm)>NiO(10-20nm)>TiO<sub>2</sub>>Fe<sub>2</sub>O<sub>3</sub>, allowing to infer that apparently Fe<sub>2</sub>O<sub>3</sub> NMs seems to be the one with less risks for receiving aquatic systems.

**Keywords:** ecotoxicity, aquatic organisms, nanomaterials, sub-lethal and lethal effects, wastewater treatment

### 2.1 Introduction

Depending on the industrial activity, resulting wastewaters can contain high loads of organic and inorganic compounds, some of them with high toxicity and

recalcitrant potential, in extremely complex mixtures. Unfortunately, for some of these wastewaters, several kind of physical, chemical, biological or a combination of treatments has been applied without satisfactory results in terms of removal rates of several contaminants, toxicity reduction or even amelioration of some physical and chemical properties (Justino et al., 2012; Kanu and Achi, 2011). However, recent European legislation namely the Water Framework Directive (WFD) (EC, 2000) extended the range of pollution control measures required to protect surface waters with a more conservative approach regarding wastewater discharges. This new approach has been driven by the improved awareness of the environmental impact of hazardous chemicals from wastewaters upon receiving ecosystems contributing for their chemical and ecological deterioration (Fatta-Kassinos et al., 2011; Kanu and Achi, 2011). Therefore, one of the main challenges to attain a good chemical and ecological status of water masses is to develop appropriate treatment of wastewaters.

In the last few years there has been a rapid development of nanotechnology and an increasing use of nanomaterials (NMs) in the manufacturing of several industrial and consumer products as well for environmental remediation purposes such as treatment of surface water, groundwater or wastewaters (Aitken et al., 2006). The increased interest in NMs is due to their unique properties such as high surface-to-volume ratio and highly reactive surface, which make them excellent adsorbents, catalysts and oxidation agents, which can be applied to degrade and scavenge pollutants (Hartmann et al., 2010; Petosa et al., 2010). Nanoscale zeolites, zero valent iron, metal oxides, carbon nanotubes and fibers and various noble metals are among NMs that have been explored for remediation (Karn et al., 2009; Shan et al., 2009).

Nanosized metal oxides, in particular, including ferric oxide, titanium oxide and nickel oxide have been the subject of intense interest. Nano-TiO<sub>2</sub> are one of the most used semiconductors for water treatment purposes due to their high photoactivity and favourable band-gap energy (Gupta and Tripathi, 2011; Velhal et al., 2012). Further, nano-TiO<sub>2</sub> activated by UV light can be applied to degrade organic compounds or even reduce toxic metal ions to a less harmful substances or to their elemental form which could be easily recovered (Guo et al., 2006; Pena et al., 2005). Nanosize iron oxides have been object of great attention due to their unique proprieties as nanosorbents

and catalysts, being a prominent photocatalytic agent able to absorb visible light wavelengths (Wang et al., 2012; Xu et al., 2012). It has been showed that nano iron oxides can degrade a real effluent from paper mill with a removal rate of 93.1% of chemical oxygen demand and also used as adsorbent of metals with an adsorption capacity higher than that reported for others adsorbents (Li et al., 2011; Nassar, 2010). Nickel oxide NPs have received considerable attention for wastewater treatment because of its chemical and magnetic properties (Khairy et al., 2012). Hayat et al. (2011), Hristovski et al. (2007) and Song et al. (2009) reported the efficient use of nano-NiO in the photocatalytic degradation of phenol, removal of metals and metalloids and in adsorption of dyes from wastewaters. In this context, although applications of NMs may bring benefits to the environment, their widespread use will also contribute for their introduction into the environment and concerns have been raised about the release of these NMs. In fact, the unique proprieties of NMs, which are responsible for their myriad applications, can also be responsible for producing ecotoxicological effects. Some NMs are photoactive and can be responsible for production of reactive oxygen species; their nanosize favours their ability to cross cell membranes and to interact with cellular components (Colvin, 2003; Li et al., 2002). Although there are some emerging literature addressing the biological effects of NMs to aquatic organisms, most of the information available are contradictory and vague (Farré et al., 2009; Krysanov et al., 2010). The most studied metal oxide is the nano-TiO<sub>2</sub> focusing on acute toxicity and mainly testing NPs around 100 nm (Ates et al., 2013; Miller et al., 2012; Nogueira et al., 2012; Song et al., 2012). However for smaller size NPs there are fewer studies, yet for water treatment applications they may be even more important, since they have higher surface areas. To the best of our knowledge, very few information is available reporting the toxicity of nano-NiO and nano-Fe<sub>2</sub>O<sub>3</sub> to aquatic organisms.

Hence, the aim of the present study was to evaluate the toxicity of four NMs: NiO with two different particle sizes (100 nm and 10-20 nm), Fe<sub>2</sub>O<sub>3</sub> and TiO<sub>2</sub> (< 25 nm), by performing a battery of tests with species from different trophic levels and functional groups. The toxicity tests were performed with the bacteria *Vibrio fischeri*, the microalgae *Raphidocelis subcapitata*, the macrophyte *Lemna minor*, the

cladoceran *Daphnia magna* and two marine species the rotifer *Brachionus plicatilis* and the crustacean *Artemia salina*. A screening test to access the mutagenicity of the NMs was also performed with two *Salmonella typhimurium* strains. This comprehensive evaluation was considered crucial to select the NM with lower risks to the environment, before assessing their potential use in the treatment of complex organic and inorganic effluents produced by industrial activities.

## **2.2 Materials and Methods**

### **2.2.1 Tested nanomaterials**

Four metal oxide NMs were tested: titanium (IV) oxide – TiO<sub>2</sub> (anatase, < 25 nm, 99.7% metal basis); two nickel oxides – NiO (100nm, 99% purity; and 10-20 nm, 99.8% purity); and iron (III) oxide – Fe<sub>2</sub>O<sub>3</sub> (nanorods: d=40-130 nm, l=250-600 nm, average particle size 85x425 nm, 99% purity). The first NM was purchased from Sigma Aldrich and the others from Nanostructured & Amorphous Materials Inc. (Houston, Texas, USA), all as nanopowders.

### **2.2.2 Characterization of aqueous suspensions of NMs**

Suspensions of the different NMs prepared with the culture media of the different test species were characterized for hydrodynamic size, polydispersity index (Pdl) and zeta potential through Dynamic Light Scattering (DLS) and Electrophoretic Light Scattering (ELS) using a Zeta sizer Nano ZS, ZN 3500, with a 532 nm laser (Malvern Instruments, UK) and coupled with the Zetasizer NanoSoftware, version 6.01. All the measurements were made at backward scattering (173°). The Pdl is a measure of the width of the distribution of particle sizes. The magnitude of the zeta potential gives an indication of the stability of NMs in the suspension, being greater for values below or above –30 mV/+30 mV, respectively (Malvern Instruments, 2008).

For size measurements, 1.5 mL of the suspension were gently transferred to a DTS 0012 polystyrene cuvette and checked for the presence of bubbles. For zeta potential measurements, 1 mL of each suspension was carefully injected with a syringe into a folded capillary cell, closed by cell stoppers and, again, the presence of bubbles



was avoided. All the measurements were made at the same temperature at which toxicity tests were done.

To assess the solubility of Ti, Fe and Ni from the nanoparticles in each medium,  $\text{TiO}_2$ ,  $\text{Fe}_2\text{O}_3$ , NiO (10-20 nm) and NiO (100 nm) suspensions at the highest concentration tested, with the corresponding culture medium, were analysed for the dissolved fraction of metals by inductively coupled plasma-mass spectrometry (ICP-MS), after acidification with nitric acid to pH 2.

The NMs suspensions were prepared by vigorously mixing the NM in the corresponding medium, through magnetic stirring, to obtain ecological relevant and homogeneous suspensions.

### **2.2.3 Ames Test with *Salmonella typhimurium***

The mutagenicity of the suspensions of NMs was assessed for two *S. typhimurium* strains (TA98 and TA100), with and without metabolic activation by the liver homogenate (S9 - Sprague-Dawley rat liver), following the procedures described by Maron and Ames (1983) and Mortelmans and Zeiger (2000) for the pre-incubation assay. Nitrofurantoin (TA100) and 2-nitrofluorene (TA98) were used as positive controls (10  $\mu\text{g}/\text{plate}$ ) in assays without S9, while 2-aminoanthracene (10  $\mu\text{g}/\text{plate}$ ) was used in assays with S9 for both strains. Sterilized distilled water was used as negative controls. The NMs were suspended in distilled water to obtain a series of dilutions to be test (1.56, 3.13, 6.25, 12.5, 25.0, 50.0 and 100%), equivalent to 0.160-10.5  $\text{mg L}^{-1}$  for NiO (100 nm), 0.390-25  $\text{mg L}^{-1}$  for NiO (10-20 nm) and 0.625-40  $\text{mg L}^{-1}$  for  $\text{Fe}_2\text{O}_3$  and  $\text{TiO}_2$ . Three replicates were considered per concentration and control. The plates were incubated for a period of 48 h, at 37 °C, and after this period the bacterial colonies formed on the plates were counted. Results were considered positive if the average number of revertant colonies was higher than twice the number recorded in negative controls or if a dose-related increase in the number of revertant colonies was observed. According to the OECD guideline for the Bacterial Reverse Mutation Test (OECD, 1997), the first criterion is sufficient for considering a positive result.

### **2.2.4 Growth tests with *Raphidocelis subcapitata***

The stock culture of *R. subcapitata* was maintained in Woods Hole MBL growth medium, at  $20\pm 2^{\circ}\text{C}$ , under continuous illumination ( $100\ \mu\text{E}/\text{m}^2/\text{s}$ ). Growth inhibition tests were conducted according to the OECD guideline (OECD, 2006a), with adaptation to 24 well microplates (Geis et al., 2000). Three replicates were established per dilution and control and each well was filled with 900  $\mu\text{l}$  of test solution plus 100  $\mu\text{L}$  of microalgae inoculum (initial cell concentration of  $10^4$  cells/ml).

The algae were exposed during 72h to several concentrations of NMs (20.0, 16.0, 12.8, 10.2 and 8.2  $\text{mg L}^{-1}$ ) plus a control (MBL medium). The tests were carried out under the same conditions used for culturing and re-suspended twice a day by repetitive pipetting to promote active gas exchange and prevent cell clumping. Cell density was estimated by measuring the absorbance at 440 nm, using a UV spectrophotometer (UV-1800, Shimadzu). In order to exclude any potential interference in the final absorbance induced by the colour of the NMs suspensions, each suspension at different concentrations was used as the respective blank at the end of the test.

### **2.2.5 Immobilization and reproduction tests with *Daphnia magna***

Monoclonal *D. magna* Straus cultures were maintained in laboratory under  $20\pm 2^{\circ}\text{C}$  and 16 h<sup>L</sup>:8 h<sup>D</sup> photoperiod, in synthetic ASTM hard water medium (ASTM, 1980), supplied with an organic additive (Baird et al., 1989). Cultures were renewed and fed with *R. subcapitata* ( $3\times 10^5$  cells/ml), every other day. Organisms used in all experiments were born between the 3rd and the 5th broods with less than 24h old.

#### **Immobilization test**

The immobilization test followed the standard procedures established by OECD protocol (OECD, 2004) for static testing conditions. For each NM five animals were randomly assigned per vessel in a total of four replicates per concentration (20.0, 16.0, 12.8, 10.2 and 8.2  $\text{mg L}^{-1}$ ) and control (ASTM). Daphnids were maintained under the same conditions mentioned for the cultures, except that no food or organic additives were added. Tests were carried in glass tubes with 25 ml of test suspensions or ASTM.

The test suspensions were prepared by mixing the NMs with ASTM medium. After the exposure period (24h and 48h), each vessel was monitored for immobilized neonates.

### **Reproduction Test**

The *D. magna* chronic tests were performed according the OECD guideline (OECD, 1998), under semi-static conditions. The concentrations tested were 0.029, 0.036, 0.045, 0.056, 0.070 mg L<sup>-1</sup> for NiO (100 nm); 0.09, 0.11, 0.14, 0.18, 0.22 mg L<sup>-1</sup> for NiO (10-20 nm); and 8.19, 10.24, 12.8, 16.0, 20.0 mg L<sup>-1</sup> for TiO<sub>2</sub> and Fe<sub>2</sub>O<sub>3</sub>. For each treatment and control, twelve individual replicates were exposed during 21 days, in 50 ml glass vials filled with 25 ml of test solution and ASTM (control), whose test medium was renewed every other day. The test conditions were the same already described for the maintenance of cultures. Animals were daily observed for offspring production and mortality. After the 21-day exposure, fecundity was determined as the total number of neonates released per female.

The rate of population increase ( $r$ , day<sup>-1</sup>) was calculated by the Euler-Lotka equation and the Jackknife technique was used to calculated standard deviation for  $r$  (Meyer et al., 1986).

#### **2.2.6 Growth test with *Lemna minor***

The macrophyte *L. minor* was maintained in laboratory under constant illumination (100  $\mu\text{E}/\text{m}^2/\text{s}$ ) at 20°C, in Steinberg medium. The 7-day *L. minor* growth test (static test) was carried out according to the OECD guideline (OECD, 2006b). The test conditions were similar to culture conditions. The tests were carried out in 150 ml Erlenmeyer's, previously sterilized, and filled with 100 ml of medium. Three colonies (similarly sized) of *L. minor* were randomly transferred to the test vessels, being the number of fronds and colonies the same in each test vessel. Three replicates per treatment and control (Steinberg medium) were exposed to different concentrations of NMs (20.0, 16.0, 12.8, 10.2, 8.2, 6.5 and 5.2 mg L<sup>-1</sup>; the last two concentrations were tested only for nano-NiO suspensions). The growth rate (GR; day<sup>-1</sup>) was determined measuring the dry weight at the beginning and after 7 days of exposure.

### 2.2.7 Microtox® Test with *Vibrio fischeri*

The bioluminescence inhibition test with *V. fischeri* was performed according to the 81.9% Basic test protocol provided by the supplier (Azur Environmental, 1998), using a Microtox toxicity analyser model 500 (Azur Environmental, CA, USA). The bioluminescence of the bacteria, exposed to several dilutions of aqueous stock suspensions (initial concentration 20 mg L<sup>-1</sup>) of the different NMs was measured after 5, 15 and 30 min of exposure.

### 2.2.8 Immobilization tests with *Artemia salina* (Artoxkit M®)

The test with *A. salina* was conducted according to the standard procedure of Artoxkit M® (MicroBioTest Inc.). The 24h bioassay was performed in a multiwell test plate using 2-day-old *Artemia* larvae (2nd instar stage). The test was conducted at 25°C and 35% salinity, with three replicates per treatment and ten animals per replicate, under dark conditions. The concentrations tested for the NMs were 0, 8.19, 10.24, 12.80, 16.00 and 20.00 mg L<sup>-1</sup>. After 24h the dead larvae in each well were counted.

### 2.2.9 Immobilization test with *Brachionus plicatilis* (Rotoxkit M®)

The test with *B. plicatilis* was performed according to the standard procedure of Rotoxkit M® (MicroBioTests Inc.). The test was conducted in multiwell plates with 300 µl per well. Six wells with five rotifers each were tested per concentration of NM (0, 8.19, 10.24, 12.80, 16.00 and 20.00 mg L<sup>-1</sup>). Incubation was done for 48h, at 25 °C, in darkness. The number of dead rotifers was registered after the exposure period.

### 2.2.10 Statistical Analysis

The EC<sub>50</sub> values, with the 95% confidence limits, for *D. magna* immobilization were calculated using a Probit analysis (Finney, 1971). For *V. fischeri* the EC<sub>50</sub> and their corresponding 95 % confidence intervals, were computed using the MicrotoxOmni® software version V1.18 with a linear model (Azur Environmental, 1998). For the growth inhibition test with *R. subcapitata* and *L. minor*, and for the chronic assay with *D. magna*, the effect concentrations (EC<sub>50</sub>, EC<sub>20</sub> and EC<sub>10</sub>) and the 95% confidence limits were calculated using the nonlinear least squares regression procedure supplied by the

software package Statistica 8.0 (StatSoft, Inc., Tulsa, USA). One-way ANOVAs followed by Dunnett tests were also employed to find out potential significant differences in the endpoints assessed between the control and tested concentrations of each NM suspension, using the software Sigmaplot version 11.0 (Systat Software, Inc., 2008; Zar, 1996). No observed effect concentration (NOEC) and low observed effect concentration (LOEC) values were taken from ANOVA significant results, reported for a significance level of 0.05.

## **2.3 Results**

### **2.3.1 Characterization of aqueous suspensions of NMs**

Table 1 displays the results of the characterization of NMs suspensions, prepared with the different test mediums, at the highest concentration tested. All the NMs showed the tendency to form large aggregates, greater than 1  $\mu\text{m}$  in size, when suspended in the different test mediums. The average hydrodynamic diameter of NMs suspended in the test media was always higher than the size reported by the manufactures. The lowest hydrodynamic diameters were recorded for nano- $\text{Fe}_2\text{O}_3$  and both nano-NiO, when suspended in Milli Q water, the medium with the lower ionic strength. Thus, the electric double layer, which helps preventing aggregation, is wider in less ionized water. The results also showed the occurrence of NMs aggregation, which is consistent with the low values of zeta potential ( $<|30\text{ mV}|$ ) for all suspensions. Further, almost all the suspension showed high values of Pdl (greater than 0.5) except once again for nano-NiO in Milli Q water suspensions (Table 1).

With respect to zeta potential measurements all the suspension displayed values within the range of -30/+30 mV, demonstrating their low stability. In saltwater media (Rotox and Artox medium) values near zero for zeta potential were recorded in some cases, which shows the high instability of NMs. Regarding particles surface charge, in a general way, all the nano-oxides presented a negative surface charge. Positive values were recorded only for nano- $\text{TiO}_2$  and nano-NiO (100 nm), but only for Microtox medium and Milli Q water or for the Microtox and Rotox medium, respectively (Table 1).

NiO NPs were the most soluble among the tested NPs in all the test medium analysed, followed by  $\text{TiO}_2$  and  $\text{Fe}_2\text{O}_3$ . In a general way the soluble fraction of metals from nanoparticles was lower in the Artox and Rotox medium. For  $\text{Fe}_2\text{O}_3$  and NiO (10-20 nm) the lowest solubility of metals was observed in Milli Q water (Table 1).

Table II.1- Data from DLS, ELS and ICP-MS for the tested NMs when suspended in all test media  
(\* Dissolved in MilliQ water)

	Medium	Conc (mg/L)	Soluble metals ions (mg/L)	pH	Zeta Potential (mv)	Cond (mS/cm)	Z average diameter (nm)	Pdl
TiO <sub>2</sub>	ASTM	20	8.23±0.07	7.84	-19.3±4.0	0.569±0.03	2271±230	0.811±0.26
	MBL	20	8.39±0.06	6.91	-22.6±4.0	0.552±0.01	1225±26	0.688±0.27
	Steinberg	20	7.46±0.06	6.01	-14.9±3.7	0.935±0.03	1605±163	0.943±0.17
	Arttox	20	7.86±0.06	8.35	-4.68±0.0	53.77±1.02	1820±103	0.789±0.24
	Rotox	20	7.82±0.07	8.53	-9.44±0.0	27.93±2.23	1707±70	0.880±0.17
	Milli Q	40	17.16±0.08	7.05	6.76±4.0	0.006±0.0	2215±91	0.962±0.07
	Microtox® solution	20	8.54±0.20*	6.57	6.21±0.0	32.78±0.32	1368±91	0.725±0.31
Fe <sub>2</sub> O <sub>3</sub>	ASTM	20	9.17±0.07	8.91	-21.4±5.9	0.566±0.003	1830±55	0.764±0.37
	MBL	20	8.75±0.04	7.03	-22.9±8.4	0.537±0.007	1545±20	0.529±0.34
	Steinberg	20	8.31±0.04	6.97	-18.8±4.9	0.903±0.01	1355±73	0.873±0.14
	Arttox	20	8.45±0.04	8.33	-10.6±0.0	54.47±0.35	2006±133	0.756±0.27
	Rotox	20	8.59±0.05	8.53	5.13±0.0	30.00±2.33	1166±51	0.834±0.18
	Milli Q	40	15.91±0.05	7.62	-15.0±5.2	0.071±0.0	524±11	0.328±0.15
	Microtox® solutin	20	7.95±0.03*	6.74	-12.5±0.0	33.30±0.26	1034±69	0.849±0.12
NiO (10-20nm)	ASTM	20	12.43±0.09	9.01	-14.3±8.7	0.626±0.01	1400±155	0.800±0.23
	MBL	20	13.51±0.07	7.19	-11.6±6.0	0.954±0.02	1266±82	0.788±0.20
	Steinberg	20	13.92±0.04	7.51	-12.4±5.5	1.047±0.03	990±74	0.851±0.17
	Arttox	20	13.72±0.04	8.40	-9.74±0.0	56.27±0.35	1243±75	0.834±0.19
	Rotox	20	13.28±0.04	8.81	-8.97±0.0	28.57±2.55	1048±94	0.874±0.14
	Milli Q	25	15.10±0.05	6.93	-17.5±4.8	0.011±0.004	659±83	0.545±0.28
	Microtox® solution	20	12.09±0.04*	8.28	-1.84±0.0	32.57±0.11	1179±81	0.878±0.14
NiO (100nm)	ASTM	20	12.94±0.04	9.50	-10.6±5.5	0.596±0.01	1126±86	0.719±0.33
	MBL	20	13.21±0.06	8.32	-14.7±5.3	0.878±0.02	2061±32	0.695±0.33
	Steinberg	20	12.29±0.05	6.60	-18.9±3.4	0.952±0.03	1910±97	0.782±0.26
	Arttox	20	12.73±0.05	8.84	-10.7±0.0	55.367±0.56	1959±102	0.690±0.34
	Rotox	20	12.47±0.03	9.33	2.48±0.0	31.680±1.56	1144±119	0.883±0.14
	Milli Q	10.5	7.05±0.02	8.85	-7.72±4.6	0.171±0.008	935±90	0.454±0.30
	Microtox® solution	20	13.44±0.03*	9.12	15.5±0.0	32.600±0.17	1877±109	0.799±0.29

### 2.3.2 Bioassays

All the tests fulfilled the validity criteria required by respective guidelines (Azur Environmental, 1998; OECD, 2006a, 2006b, 2004, 1998, 1997).

All the NMs screened revealed no direct or indirect mutagenicity for both *S. typhimurium* strains.

For the bioassays with marine species no toxicity was observed for any of the NM's suspensions in the range of concentrations tested.

The results obtained in the growth inhibition test with *R. subcapitata* showed no significant inhibitory effects for nano-Fe<sub>2</sub>O<sub>3</sub> (Fig. 1). In opposition, the growth rate was significantly reduced by the two nano-NiO, with EC<sub>50</sub> values of 8.24 mg L<sup>-1</sup> for NiO (100 nm) and 15.2 mg L<sup>-1</sup> for NiO (10-20 nm) (Table 2). However, for nano-TiO<sub>2</sub>, a statistic significantly stimulation occurred in the growth rate of *R. subcapitata* for the two highest concentrations (16 mg L<sup>-1</sup> and 20 mg L<sup>-1</sup>).

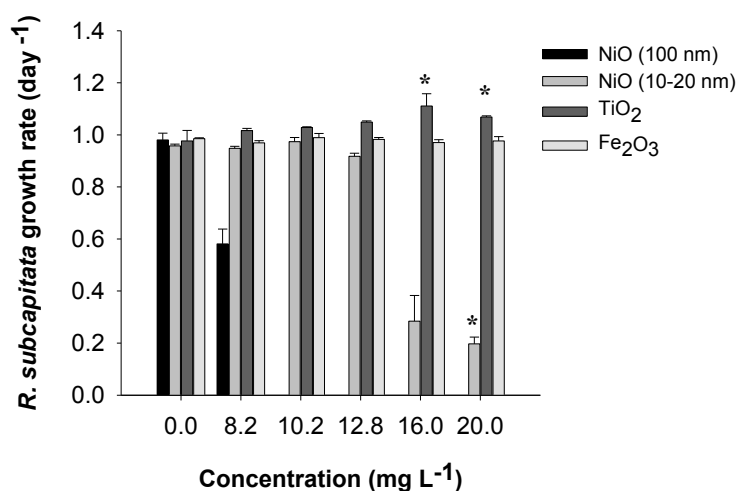


Figure II.1- Growth rate (day<sup>-1</sup>) of *R. subcapitata* exposed to the four NMs. Error bars: standard error; asterisks: statistically significant differences from the control.



Table II.2- Ecotoxicity data for nano-NiO (100 nm) and nano-NiO (10-20 nm) with corresponding 95% confidence intervals.

		NiO 100nm			NiO 10-20nm		
		EC <sub>50</sub> /LC <sub>50</sub> (mg L <sup>-1</sup> )	LOEC (mg L <sup>-1</sup> )	NOEC (mg L <sup>-1</sup> )	EC <sub>50</sub> /LC <sub>50</sub> (mg L <sup>-1</sup> )	LOEC (mg L <sup>-1</sup> )	NOEC (mg L <sup>-1</sup> )
<i>R. subcapitata</i>	Growth inhibition	8.25 (-5.6-22.13)	10.2	8.19	15.2 (14.5-15.8)	20.0	16.0
<i>D. magna</i>	Immobilization						
	24h	14.6 (13.1-16.7)	-	-	13.98 (12.7-15.6)	-	-
	48h	9.74 (7.73-11.1)	-	-	9.76 (8.53-10.7)	-	-
	Fecundity	-	0.05	0.04	-	0.14	0.11
	Rate of population increase	-	-	-	-	-	-
<i>L. minor</i>	Growth inhibition	4.39 (3.02-5.76)	5.24	-	-	8.19	6.55

Both nano-NiO were also toxic to *L. minor*, contributing for a significant reduction in the growth rate. LOEC values of 5.25 and 8.19 mg L<sup>-1</sup> were recorded for NiO (100 nm) and (10-20 nm), respectively (Table 2), whilst for nano-TiO<sub>2</sub> and nano-Fe<sub>2</sub>O<sub>3</sub> no significant effects were observed within the range of concentrations tested (Fig. 2).

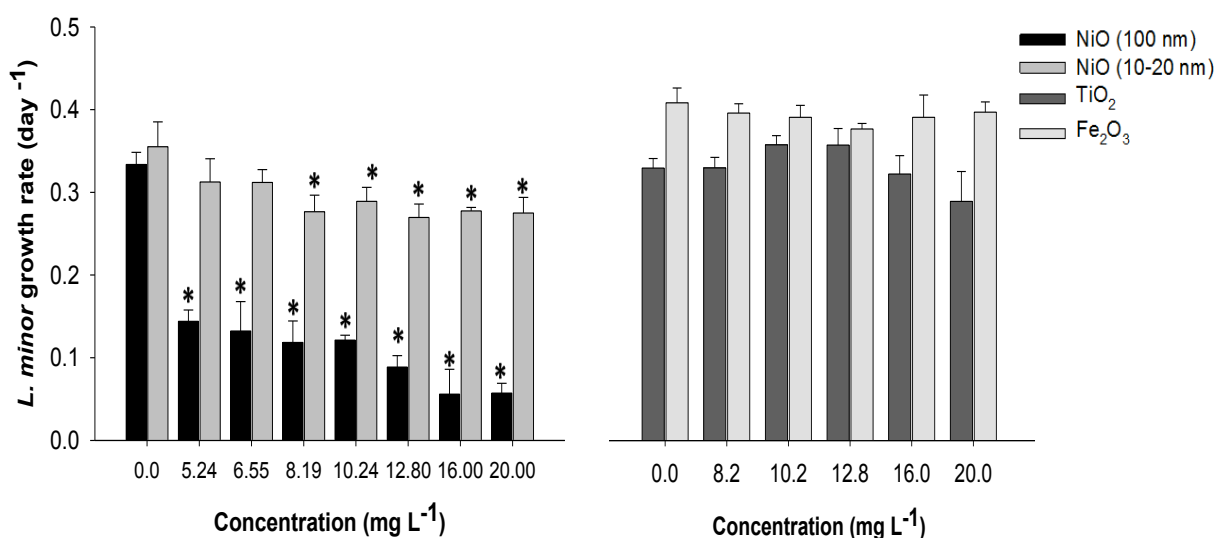


Figure II.2- Growth rate (day<sup>-1</sup>) of *L. minor* exposed to the four NMs. Error bars: standard error; asterisks: statistically significant differences from the control.

In what concerns *D. magna*, both nano-NiO were acutely toxic to this species, with EC<sub>50</sub> ranging from 9.74 mg L<sup>-1</sup> (24h) to 14.6 mg L<sup>-1</sup> (48h) (Table 2). As far as sub-

lethal effects are considered, in the 21-day reproduction test, the fecundity was significantly affected with LOEC values ranging from 0.045 to 0.14 mg L<sup>-1</sup> (Table 2 and Fig. 4). Regarding nano-TiO<sub>2</sub> and nano-Fe<sub>2</sub>O<sub>3</sub> no lethal effects were observed for *D. magna*, but in the chronic test a LOEC value of 16.0 mg L<sup>-1</sup> was recorded for TiO<sub>2</sub> (Fig. 3) for fecundity. The intrinsic growth rate of population (*r*) was not significantly affected for both nano-NiO and for nano-Fe<sub>2</sub>O<sub>3</sub> but for the nano-TiO<sub>2</sub> was recorded a LOEC value of 16 mg L<sup>-1</sup>.

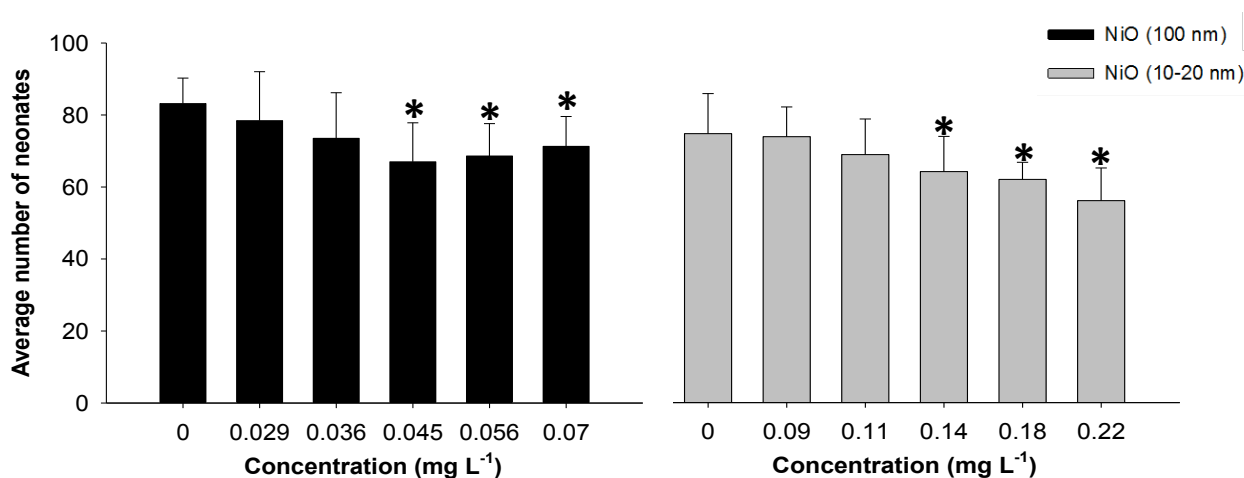


Figure II.3- Number of offspring produced during 21 days by *D. magna* at different concentrations of NiO (100 nm) and NiO (10-20). Error bars: standard error; asterisks: statistically significant differences from the control.

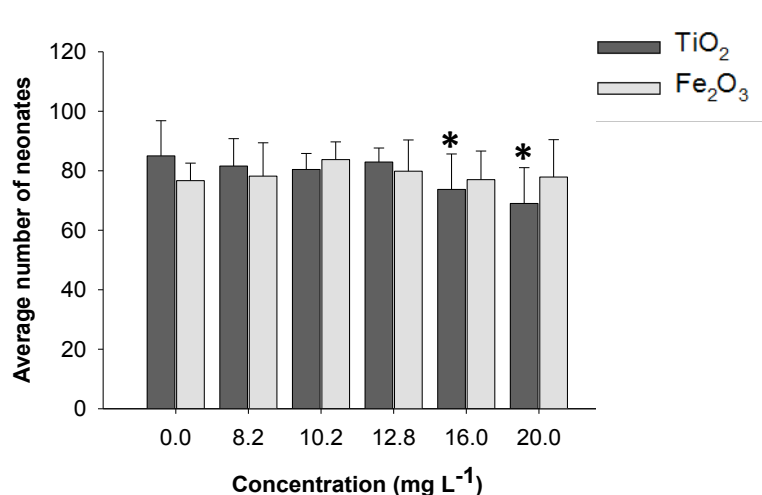


Figure II.4- Number of juveniles produced during 21 days by *D. magna* at different concentrations of TiO<sub>2</sub> and Fe<sub>2</sub>O<sub>3</sub>. Error bars: standard error; asterisks: statistically significant differences from the control.

## 2.4 Discussion

The stability of nanoparticles is a function of many factors including pH and ionic concentration of the surrounding environment. As a consequence, the stability of NPs in a particular medium is expected to affect its toxicity, mobility and bioavailability. Also the physical and -chemical characteristics of the medium can affect the agglomeration state and surface charge. In our study almost all the NPs present a negative charge with high aggregation state. For example, Jiang et al. (2009) demonstrated that increasing ionic strength from 1-100 mM led to a 50-fold increase in the hydrodynamic diameter for TiO<sub>2</sub> dispersions (15nm). However, despite zeta potential decrease with increasing ionic strength the surface charge of TiO<sub>2</sub> was unchanged. The same study also reported that changing the pH of the solution can alter both particle surface charge and hydrodynamic size. The nanoparticle surface charge depends of its isoelectric point (zero surface charge), hence at high pH the particles have a negative surface charge and at low pH a positive surface charge. Also it was expected that positive charge nanoparticles will bind to negatively charged cell surface and be absorbed more efficiently than negatively charged particles and so promote more toxicity to the cell. However this was not observed in our study, and in the literature there are other reports showing the same evidence; i.e. the uptake of negatively charged particles despite the unfavourable interaction between the particles and the cell membrane (Verma and Stellaci 2010). In our study, as it will be discussed herein other parameters seemed to have a more meaningful role in the toxic effects observed than the surface charge of the NPs.

All the NMs screened revealed no mutagenicity for both *S. typhimurium* strains (TA98 and TA100). These results were in agreement with those reported by other authors, which till now have recorded no mutagenicity potential of different nanosize metal oxides (Al<sub>2</sub>O<sub>3</sub>, Co<sub>3</sub>O<sub>4</sub>, TiO<sub>2</sub>, ZnO and Fe<sub>3</sub>O<sub>4</sub>) to *S. typhimurium* and *E. coli*, both in the absence and presence of metabolic activation (Lopes et al., 2012; Pan et al., 2010; Pereira et al., 2011; Szalay et al., 2012). However, although the nano-TiO<sub>2</sub> tested in this study was smaller (25 nm) than those used in previous studies, their size did not favour the cellular uptake and access to genetic material. A recent review made by Doak et al.

(2012) show that in 19 studies published, where the Ames test was used for the genotoxicological analyses of NMs, 17 had a negative response and the other two reported a weak mutagenic effect. However when other in vitro mammalian cell test systems are performed (e.g. chromosomal aberration, micronucleus or comet assay) they have been found to have a positive genotoxic response. This phenomenon was related to the degree of the NM uptake by the bacterial cells and their inability to cross the cell wall, which is likely less than in mammalian cells, and also a consequence of particle aggregation (Doak et al., 2012). Nevertheless, after the sonication and filtration of NPs suspensions Baek and An (2011) showed that CuO, NiO, ZnO, and Sb<sub>2</sub>O<sub>3</sub> exhibit antibacterial activity against *E. coli*, *Bacillus subtilis* and *Streptococcus aureus*. However in environmental conditions nanoparticles will be probably more aggregated and will not cross the cell wall and the exposure concentrations tested were higher than ours. In fact, according to our results (Table 1) the aggregation and sedimentation of NPs decreased the solubility of its metals ions in Milli Q water (dilution medium in the Ames test), especially for Fe<sub>2</sub>O<sub>3</sub> and NiO (10-20 nm).

In the assays with the marine species (*V. fischeri*, *A. salina* and *B. plicatilis*) no toxicity was observed for all the compounds tested, likely due to the high ionic strength of the seawater. In a general way, NMs displayed the lowest absolute values of zeta potential and high hydrodynamic diameters and low metal's solubility (Table 1) when suspended in seawater medium, indicating the lower stability in this medium and their tendency to aggregate and settle, becoming less accessible for biosorption/uptake by test species. Our results were coincident with those from Keller et al. (2010), reporting the electrophoretic mobility, aggregation and rate of sedimentation of three metal oxide NMs dispersed in seawater and freshwater mediums. The results showed that in the seawater, the rate of sedimentation was very high for all the NMs, and was concentration-dependent. This agrees with the expected decrease of the electric double layer around the NMs, when using seawater, which favours particle aggregation, more so when particle concentration increases. On the other hand, the same authors observed that in the freshwater medium the size of NM aggregates remained stable (Keller et al., 2010). Positive surface charges of some nano-oxides, also did not contribute for their toxicity to *V. fischeri*. The change

observed in some cases (e.g. for nano-TiO<sub>2</sub> and NiO in the Microtox medium) was probably due to the isoelectric point of these particles. When the ionic content of the medium increases surface charge can be easily reversed, as it is very sensitive to the concentration, pH and type of ions in solution (Malvern Instruments, 2008). Our results are consistent with those reported for *A. salina* by Ates et al. (2013), since they observed that a rapid aggregation occurred in all concentrations tested (10 to 100 mg L<sup>-1</sup>) in seawater medium and, despite the accumulation of aggregates by both larvae and adults, no significant mortality was recorded within 24h. Further, after 96h the LC<sub>50</sub> was higher than 100 mg L<sup>-1</sup> (Ates et al., 2013).

In opposition, the freshwater species (*R. subcapitata*, *L. minor* and *D. magna*) were acutely and chronic affected by almost all the nano-oxides suspended in their media. In the 72h growth inhibition test with *R. subcapitata* no significant effects were observed for Fe<sub>2</sub>O<sub>3</sub>, in opposition to both NiO (100 nm and 10-20 nm) (Fig. 1). In recent studies, Gong et al. (2011) and Han and Zhang (2012) tested the effect of NiO on algae, recording a 72h-EC<sub>50</sub> value of 32.3 mg L<sup>-1</sup> and a 96h-EC<sub>50</sub> of 40.8 mg L<sup>-1</sup> for *Chlorella* growth. In our study even lower EC<sub>50</sub> values were recorded for *R. subcapitata* exposed to NiO of both sizes: 8.25 mg L<sup>-1</sup> (100 nm) and 15.2 mg L<sup>-1</sup> (10-20 nm). However, these EC<sub>50</sub> values are remarkable higher than the range of concentrations (100-700 µg L<sup>-1</sup>) reported as inhibiting the growth of four freshwater algae species, for non-nano Ni (Eisler, 1998). This suggested that probably were the smaller NMs that persist in the medium that promote the toxic effects on algae. A fact that can be corroborated with the high values of Pdl (Table 1) which indicate non-homogeneous suspensions with a broad particle size distribution. In summary, not only the average hydrodynamic size of the NMs, but also the distribution of sizes in the suspensions should be analysed to predict their toxicity since, the aggregation of NMs in the environment may apparently reduce their toxicity to values lower than those recorded for non nanosize parental compounds. Also, both NiO were the most soluble in the MBL medium indicating the presence of high concentrations of the ionic form of nickel in solution when compared to TiO<sub>2</sub> and Fe<sub>2</sub>O<sub>3</sub> (Table 1). Therefore, the toxic effects recorded for NiO were likely related with the highest solubility of Ni ions. Regarding the nano-TiO<sub>2</sub> our results contradict the majority of the studies with *R. subcapitata*, which recorded toxic effects

for concentrations ranging from 5.83 mg L<sup>-1</sup> to 241 mg L<sup>-1</sup> (Menard et al., 2011). Kulacki and Cardinale (2012), have also recorded a positive effect of nano-TiO<sub>2</sub> (25 nm), on maximum algae biomass yield, at a range of concentrations well above the one tested in our study (50-300 mg L<sup>-1</sup> of nano-TiO<sub>2</sub>), testing ten different freshwater species. However, the positive effect was recorded especially for the highest concentrations and the possible explanation (nano-TiO<sub>2</sub> could reduce the competition for nutrients killing the bacteria) proposed by these authors are not applicable to our results, since we have performed the assays under axenic conditions, using the nutrient-rich MBL medium. Further, this stimulation is particularly curious, because the adsorption of NMs to the cells surface and the subsequent block of electrolytes and metabolites transport across the membrane was suggested as the primary mechanism of nano-TiO<sub>2</sub> toxicity to algae (Metzler et al., 2012).

For *L. minor* no significant effects were observed for TiO<sub>2</sub> and Fe<sub>2</sub>O<sub>3</sub> (Fig. 2), at least at the concentrations tested. However, despite no information is available about the concentrations of these NMs in the environmental compartments, in our opinion concentrations above this level will have no ecological relevance. In fact, Kim et al. (2011) and Song et al. (2012) found that nano-TiO<sub>2</sub> can cause growth inhibition in two species of *Lemna* but the concentrations tested were much higher than those tested in our study. Further, these authors have tested nano-TiO<sub>2</sub> with 2-3 nm and 5-10 nm in size, what may explain the difference in toxicity results. Pinheiro et al. (2013), using nuclear microscopy, found no internalization of nano-TiO<sub>2</sub> (average size 28±11 nm) by *L. minor* at the maximum concentration tested (25 mg L<sup>-1</sup>), despite the accumulation of Ti in the lower surface of fronds and in the roots. The lack of internalization, due to particles aggregation (Table 1), was likely the reason for the absence of toxicity on the macrophytes exposed for 7 days, which is near the maximum concentration tested in this study. The same explanation was suggested by Li et al. (2013). Iron is also an essential element to plants which have developed several mechanisms for regulating iron homeostasis (Briat et al., 2010). These mechanisms have probably worked to prevent the toxicity of nano-Fe<sub>2</sub>O<sub>3</sub> to duckweeds and also to *R. subcapitata*. Regarding nano-NiO, with 100 nm and 10-20 nm, we recorded toxic effects on *L. minor* with LOECs values of 5.24 and 8.19 mg L<sup>-1</sup> (Table 2). To the best of our knowledge, we did

not find any study regarding the toxicity of nano-NiO with this macrophyte, but it is known that nickel (Ni), although being an essential micronutrient at high concentration it has proved to be phytotoxic (Brown et al., 1987). Khellaf and Zerdaoui (2010) and Drost et al. (2007) showed that higher concentrations of Ni can inhibited the growth of *L. minor* reporting EC<sub>50</sub> of 1.29 mg L<sup>-1</sup> and 3.30 mg L<sup>-1</sup>, respectively. Once again, these results suggest that the higher solubility of nano-NiO, compared to the other NMs, favour the uptake of Ni. Being an essential element, the adsorption of nano-NiO could have been carried out by regular mechanisms used by plants to adsorb these elements, like the production of chelating agents, plant-induced changes in pH and redox reactions, which are able of solubilize micronutrients even from insoluble precipitates (Tangahu et al., 2011).

Regarding *D. magna* the findings suggested that exposure duration can contribute for increasing toxicity of these NMs, since for both NiO and TiO<sub>2</sub> was verified an increase in toxicity when the time of exposure rises, from the 48h-acute test to the 21d-reproduction test. Unfortunately, no literature values have been found for NiO to compare these results. Zhu et al. (2010) also found that in an acute 48h test nano-TiO<sub>2</sub> (particle size 21 nm) exhibited minimal toxicity (EC<sub>50</sub>>100 mg L<sup>-1</sup>) but when they extended the test for 72h its toxicity has remarkably increased (EC<sub>50</sub>=1.62 mg L<sup>-1</sup>). These authors also reported sub-lethal effects in *D. magna* after a 21-day exposure. The results of our study showed that *D. magna* was the most sensitive species, recording the lowest EC<sub>50</sub> and LOECs values for both nano-NiO and nano-TiO<sub>2</sub>. This crustacean is widely used as test organism for ecotoxicological studies, they are filter feeders and can filter relatively large amounts of water compared to its body size being able to ingest particles in the size range from 0.6 to 40 µm (Geller and Müller, 1981), so they were likely exposed to a large number of NMs and NMs-aggregates over the exposure time. Similarly Griffitt et al. (2008) found that *D. pulex* was the most sensitive organism for nanosize silver and copper. Based on the study of Fouqueray et al. (2012), during chronic exposures the adsorption of NMs on algae surface, used to feed the organism, could also enhance their uptake by cladocerans, explaining the effects recorded. Additionally, to the possible internalization of Ti, in the gut tissues and the subsequent chemically-induced toxic, as observed by Pinheiro et al. (2013) in *D. magna*

exposed for 7 days to  $1.4 \text{ mg L}^{-1}$  of  $\text{TiO}_2$ , other studies suggested different mechanisms of NMs toxicity on cladocerans, which may explain their enhanced sensitivity. Dabrunz et al. (2011), observed a surface coating on *D. magna* neonates exposed to nano- $\text{TiO}_2$  ( $2 \text{ mg L}^{-1}$ ;  $\approx 100 \text{ nm}$ ), which has compromised the second molting, reducing mobility and ultimately causing mortality after 96h. In our study, although no toxic effects of nano- $\text{Fe}_2\text{O}_3$  have been observed, its dark colour allowed to confirm this biological coating phenomenon (author's personal observation). However, the absence of chronic effects on *D. magna* for this NM, suggest that a combination of both chemical and physical induced mechanisms are responsible by the ecotoxicological effects of NMs to aquatic species. García et al. (2011) also tested the effect of iron oxide nanoparticles ( $\text{Fe}_3\text{O}_4$ ) to *D. magna* recording a  $\text{LC}_{50}$  of  $0.0023 \text{ g L}^{-1}$ , However, in this study, the solution of  $\text{Fe}_3\text{O}_4$  NPs were stabilized to prevent agglomeration. Without the agglomeration  $\text{Fe}_3\text{O}_4$  were probably better internalized by *D. magna* promoting the recorded toxic effect (García et al., 2011). Although meaningful in reporting the true toxicity of these NM, the results of these previous authors may lack ecological relevance, since all the data available for the characterization of NPs suspensions, made us to expect that the non-aggregated form will be the less dominant form of NPs in the aquatic environment.

According to all the results, nano-NiO were the most toxic for the freshwater aquatic species tested. This fact seems to be associated to the solubility of the tested NMs in the respective medium, since both NiO exhibit high solubility compared to nano- $\text{TiO}_2$  and nano- $\text{Fe}_2\text{O}_3$ . However, in opposition to what was expected the toxicity of NiO did not increase when size decreases, since nano-NiO (10-20 nm) was consistently less toxic. This could be explained by different aggregation behaviours and by the values of Pdl. Both nano-NiO recorded high values of Pdl in all the test mediums, and assuming that in the case of nano-NiO (100 nm) we have a greater diversity of size aggregates, the few small aggregates that are formed could be enough to induce toxic effects. It is known that for the same zeta potential the smaller particles tend to be less stable due to the higher Gibbs surface energy, thus leading more easily to the formation of irreversible aggregates. Another possible speculation is related to the effective interaction area of a large particle (nano-NiO 100 nm) relatively



to the small particles (nano-NiO 10-20 nm). Within this specific area, an individual large particle has more functional groups to exert effects on the cells. But if we consider an equal mass of small and large particles, the large number of small particles increases the number of points of interaction with the cell. Probably in our study, due to the lower number of smaller particles, as a result of aggregation, each interaction point exerts a more weak effect on the cells comparatively to the large particles of NiO (100 nm).

## 2.5 Conclusions

From the results of this study we can establish a decreasing order of toxicity  $\text{NiO}(100\text{ nm}) > \text{NiO}(10\text{-}20\text{ nm}) > \text{TiO}_2(<25\text{ nm}) > \text{Fe}_2\text{O}_3(\approx 85 \times 425\text{ nm})$  and the most sensitive organism was *D. magna*. Once in aquatic media with different physical and chemical parameters, NMs may behave differently than expected according to indications given by manufactures, since their surface charge may change, and may form aggregates of different size. Such unexpected changes in behaviour indicate that it is difficult to generalize relationships between their reported properties and their toxic potential. Further, other biotic parameters may influence the exposure to different nanomaterials, contributing for species-specific sensitivities. No apparent relationship was found between surface charge, zeta potential, size and toxicity for the organism tested, however the toxicity differed with the particle chemical composition and solubility. Although it has been reported in the literature, the NMs with lower size did not promote the highest toxicity in this study. So according to the results, apparently  $\text{Fe}_2\text{O}_3$  and  $\text{TiO}_2$  NMs seemed to be the ones with less risks to the receiving aquatic systems, if accidental releases to the environment occur after being used in effluent treatment plants. Therefore, further studies are required having in consideration the potential aggregation of these NMs, and their settlement in the sediments compartment, in order to test its toxicity to benthic fauna. The risk of using NMs for remediation purposes can be probably negligible for the marine organisms since the marine ecosystem is not the primary receiving environment for this wastes and also because we did not record any toxic effect in these organisms. Further cumulative

effects of discharges in parallel with the fate of NM aggregates in the environment have to be evaluated as well.

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# Chapter III

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Photocatalytic treatment of olive oil mill wastewater using  $\text{TiO}_2$  and  $\text{Fe}_2\text{O}_3$  nanomaterials



## Photocatalytic treatment of olive oil mill wastewater using TiO<sub>2</sub> and Fe<sub>2</sub>O<sub>3</sub> nanomaterials

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### Abstract

Olive oil industry produces a highly polluted wastewater, which represents an important environmental problem in the Mediterranean region. Several physicochemical, biological and combined processes have been used but none of these treatments were totally effective in mitigating their toxicity and subsequently impacts in the receiving systems. In this context, nanotechnology arises offering new possibilities for the treatment of wastewaters mainly based on the enhanced physical and chemical properties of nanomaterials (NMs), which can remarkably increase their adsorption and oxidation potential. The aim of this work was to investigate the treatability of OMW through several systems involving advanced oxidation processes (UV/H<sub>2</sub>O<sub>2</sub>, UV/TiO<sub>2</sub>, UV/Fe<sub>2</sub>O<sub>3</sub>, UV/TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub> and UV/Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub>) and using as catalysts two nanomaterials (TiO<sub>2</sub> and Fe<sub>2</sub>O<sub>3</sub>). The effects of catalyst dose and oxidant concentration were also investigated. Ours results suggest that photodegradation systems associating TiO<sub>2</sub> or Fe<sub>2</sub>O<sub>3</sub> with H<sub>2</sub>O<sub>2</sub> were the most efficient treatments. In terms of toxicity to *V. fischeri* we recorded reductions for the systems UV/TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub> and UV/Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub>, although the highest reduction was recorded in the treatment with H<sub>2</sub>O<sub>2</sub>/UV. In this work the use of nanomaterials combined with H<sub>2</sub>O<sub>2</sub> showed a great potential in removing phenols, the major toxic compounds of this wastewater.

**Keywords:** Olive mill wastewater, nanomaterials, photocatalytic degradation

### 3.1 Introduction

Olive oil extraction is an economically important activity in the Mediterranean region and, according to statistics of the International Olive Oil Council from November 2013, the production of this agriculture product continues to increase every year and is no longer restricted to the Mediterranean area (IOC, 2013). The extraction process that operates seasonally requires an high volume of water, and consequently

generates over 30 million m<sup>3</sup> of oil mill wastewater each year (Ahmadi et al., 2005; Kavvadias et al., 2010). Olive oil mill wastewater (OMW) is one of the most concerning agro-industrial wastes. The physicochemical characteristics of OMW depends on the variety of olives and its degree of maturation, the climatic conditions and the method of extraction (Hanafi et al., 2010a; Justino et al., 2010; Roig et al., 2006). The OMW, a dark-colored waste, is highly rich in organic compounds such as sugars, polyphenols, lipids, organic acids and pectin. The phenolic content, the high COD (Chemical Oxygen Demand) and BOD (Biochemical Oxygen Demand) levels and the toxic and phytotoxic properties of this effluent make its degradation a difficult and expensive process (Justino et al., 2012; Paraskeva and Diamadopoulos, 2006). Despite that, OMW has been disposed untreated in the environment into rivers, in the sea, in soil or in lagoons/ponds (Kavvadias et al., 2010; Saadi et al., 2007).

In order to deal with this problem, numerous processes have been developed to treat OMW in order to reduce its environmental impact, like physicochemical (e.g. ozonation, coagulation, ultrafiltration, electrochemical oxidation) and biological approaches (e.g. aerobic or anaerobic biological treatments) as well as combined technologies. Although with a certain degree of effectiveness, none of these treatments were totally efficient in mitigating the toxicity of OMW and the costs involved in the process are still relatively high (Mantzavinos and Kalogerakis, 2005; McNamara et al., 2008; Roig et al., 2006). Currently, the most cost-effective solution being widely used is the storage in evaporation ponds, but the environmental impact still persists (Kavvadias et al., 2010).

Therefore, in order to reduce the risk associated with the release of OMW to aquatic ecosystems, it is imperative to develop new technologies capable of promoting the effective degradation of its recalcitrant organic compounds. A promising way to accomplish the mineralization of persistent organic pollutants is the application of advanced oxidation processes (AOPs) (Oller et al., 2011; Poyatos et al., 2010). Advanced oxidation processes include several methodologies like photolysis, ozonation, Fenton reaction, ultrasounds, wet air oxidation and photocatalysis, based on the production *in situ* of highly reactive non-selective free radicals (Cañizares et al., 2007b; Stasinakis, 2008). Photocatalysis is an emerging technology of the AOPs which

combines semiconductors (e.g., TiO<sub>2</sub>, ZnO), light and/or oxidants, in the total degradation of organic contaminants into H<sub>2</sub>O and CO<sub>2</sub> (Ahmed et al., 2010; Cañizares et al., 2007b; Mantzavinos and Kalogerakis, 2005; Poyatos et al., 2010; Stasinakis, 2008). The excitation of the semiconductor with energy equal or greater than its band gap promotes the release of electrons (e<sup>-</sup>) from the valence band into the conduction band, leaving a positive hole (h<sup>+</sup>) in the valence band. *Hole* and electron pairs can migrate to the surface of catalyst promoting oxidation-reduction reactions with the adsorbed pollutants (Ahmed et al., 2010; Hoffmann et al., 1995).

In order to achieve a maximum yield on oxidation reactions, nanoparticles (NPs) are being applied as catalysts in photocatalytic systems. Nano-scale semiconductor particles have high surface to volume ratio that greatly increases the density of active sites available for adsorption and catalysis. Moreover, with the decrease of the particle size, the band-gap energy increases greatly, leading to higher redox potentials and less recombination of the electron-hole pair (Curri et al., 2003; Dai et al., 2007; Hoffmann et al., 1995; Khin et al., 2012; Liu et al., 2005; Savage and Diallo, 2005; Theron et al., 2008). Recent studies recognized that the NP form of TiO<sub>2</sub> is much more efficient (enhanced photocatalytic activity) when compared to its bulk counterpart (Han and Bai, 2009; Swetha et al., 2010). Moreover, it has been also proved the ability of these nanomaterials to degrade organic compounds (Guo et al., 2006; Li et al., 2011; Nagaveni and Sivalingam, 2004; Savage and Diallo, 2005; Xu et al., 2012). Based on these previous studies we have investigated the treatability of OMW through different treatment strategies involving AOPs, using two NP as catalysts, TiO<sub>2</sub> and Fe<sub>2</sub>O<sub>3</sub>. Both semiconductors were chosen since they are non-toxic to both freshwater and marine species (Hund-Rinke and Simon, 2006; Ko and Kong, 2013; Nogueira et al., 2014; Wiench et al., 2009) and are relatively cheap and commercially available (Chirita and Grozescu, 2009; Ghaly et al., 2011). Although there are some reports showing the applicability of these two NPs in photocatalysis, most of the studies are in individual compounds, and the great majority of them deal with dyes (Delgado, 2010; Giwa et al., 2012; Jagadale et al., 2012; Jahagirdar et al., 2011; Khattab et al., 2012; Lima et al., 2014). Nonetheless, only one study described the application of synthesized nano TiO<sub>2</sub> and solar photocatalysis to treat a paper mill effluent (Ghaly et al., 2011). To the best

of our knowledge, there are no studies reporting the photochemical degradation of a real OMW involving these two NPs.

The photodegradation process was carried out using combinations of treatments such as hydrogen peroxide with UV (UV/H<sub>2</sub>O<sub>2</sub>), TiO<sub>2</sub> and Fe<sub>2</sub>O<sub>3</sub>-assisted photo-catalysis (UV/TiO<sub>2</sub> and UV/Fe<sub>2</sub>O<sub>3</sub>), UV/TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub> and UV/Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub> systems. The effect of catalyst (0.25, 0.50, 0.75, 1.0 and 2.0 g L<sup>-1</sup> of TiO<sub>2</sub> and Fe<sub>2</sub>O<sub>3</sub>) and oxidant concentrations (0.03, 0.06, 0.13, 0.25, 0.50 and 1.00 M) were also investigated to establish optimal conditions to treat the OMW. The efficiency of the treatment was assessed through aromatics (270nm) and colour reduction (465nm), chemical oxygen demand (COD), total phenolic content and ecotoxicity (Microtox®).

## **3.2 Materials and Methods**

### **3.2.1 Olive oil mill wastewater and reagents**

The sample of OMW was collected in the north of Portugal from an evaporation pond and it was the by-product of a stepwise extraction procedure (three-phase olive oil extraction) in a small-scale production facility. The olive oil mill wastewater sample was collected in screw-cap glass flasks, which were loosely capped and immediately transported to the laboratory, where they were stored at 4°C until treatment. The effluent had a dark black colour, inherent odour and contained a substantial fraction of aromatic compounds, which was consistent with a characteristic absorbance peak at 270 nm. The effluent's major properties were as follows: COD= 33.6±0.6 g L<sup>-1</sup>, total phenolic content= 243±14 mg L<sup>-1</sup> and pH 4.0±0.1. This effluent presented large amounts of phenols as well acidic pH, which contributed for characterizing OMWs as a recalcitrant effluent. However the original OMW was filtered with glass fiber filters and diluted 25% with tap water before carrying out the photocatalytic experiments (COD= 7.0±1.0 g L<sup>-1</sup>; total phenolic content= 42.6±5.6; pH 4.4±0.1).

The two NPs were purchased as nanopowders; titanium (IV) oxide – TiO<sub>2</sub> (anatase, particle size < 25nm, 99.7% metal basis) from Sigma Aldrich and iron (III) oxide – Fe<sub>2</sub>O<sub>3</sub> (nanorods, particle information: d=40-130nm, l=250-600nm, average particle size 85x425 nm, 99% purity) from Nanostructured & Amorphous Materials Inc.

(Houston, Texas, USA). Hydrogen peroxide (30%, analytical grade) was purchased from Fisher Scientific.

### 3.2.2 Photocatalytic experiments

Photodegradation treatments were carried out using 50 ml of OMW (25%) in 100 ml glass beakers, maintained at 20°C. No adjustments in pH were made since in the literature the optimum pH for photodegradation processes was reported to be between 2 and 5 (Chatzisyneon et al., 2008; Ferreira et al., 2008; Kallel et al., 2009). Three replicates were established per treatment. The suspensions were placed on an orbital shaker (100 rpm) for a period of 24h to attain adsorption–desorption equilibrium regarding the organic compounds on the catalyst surface. After this period the UV lamp (Spectroline XX15F/B, Spectronics Corporation, NY, USA, peak emission at 312 nm) was switch on, and left for 2 hours. During irradiation, agitation was maintained by magnetic stirring to keep the suspension homogenous. The influence of different concentrations of nano-TiO<sub>2</sub> and nano-Fe<sub>2</sub>O<sub>3</sub> was studied (0.25, 0.50, 0.75, 1.0 and 2.0 g L<sup>-1</sup>). According to the literature, the presence of strong oxidant species can greatly enhance the photodegradation process (Chiou et al., 2008b; Garcia et al., 2007; Ghaly et al., 2001; Jamil et al., 2011). Therefore the influence of different concentrations of H<sub>2</sub>O<sub>2</sub> was also investigated (0.03, 0.06, 0.13, 0.25, 0.50 and 1.00 M). In the UV/TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub> and UV/Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub> systems, to assess the influence of different H<sub>2</sub>O<sub>2</sub> concentrations, the concentration of the NPs was fixed in 1.0 g L<sup>-1</sup> of nano-TiO<sub>2</sub> or nano-Fe<sub>2</sub>O<sub>3</sub> and different volumes of H<sub>2</sub>O<sub>2</sub> were tested. After the UV exposure the samples were centrifuged (14000 rpm for 20 min) and filtered through GF/F (Ø47mm, Whatman) glass-fiber filter to remove the catalyst particles before analysis. In the treatments with H<sub>2</sub>O<sub>2</sub>, samples were withdrawn for analysis of chemical oxygen demand (COD) and for the ecotoxicity tests after the addition of sodium hydroxide (0.6 N) and heating in a bath (100°C) for 4h to completely eliminate residual hydrogen peroxide. It has been shown that the rate of H<sub>2</sub>O<sub>2</sub> decomposition (to oxygen and water) increases several folds as pH increases and temperature rises (Qiang et al., 2002).

### 3.2.3 Analytical determinations

The absorbance measurements, before and after treatments, were made by UV/VIS spectrophotometry (Shimadzu 1800) at the wavelengths associated with aromatic (270 nm) and colored (465 nm) compounds. The COD decrease was determined following American Society for Testing Materials standard method D 1252-88 (ASTM, 1994), pH was measured with a pH meter (pH meter 330 from WTW), and total phenolic content was determined by colorimetric method of Folin–Ciocalteu (Mulinacci et al., 2001).

### 3.2.4 Microtox assays

The ecotoxicity of all the effluent samples, before and after the treatments, was assessed following the 81.9% Basic Test protocol provided by the supplier, using a Microtox Model 500 Analyser (Azur Environmental, 1998). The endpoint measured in the Microtox assay was the decrease in the intensity of light emitted by the luminescent marine bacteria – *Vibrio fischeri* – after 5, 15 and 30 min of exposure to several dilutions of the effluent (using a dilution factor of 2x), after previous osmotic and pH (6-8) adjustment of the samples.

### 3.2.5 Statistical analysis

The EC<sub>50</sub> values and their corresponding 95% confidence intervals for *V. fischeri*, were calculated using the MicrotoxOmni® software version V1.18 for the three exposure periods (Azur Environmental, 1998). To test for statistically significant differences among the raw data recorded for each parameter analysed (colour, aromatic compounds, COD and total phenolic content) under different treatments and control (non-treated effluent), a parametric one-way analysis of variance (ANOVA) was employed, followed by Dunnett's test (a level of significance of 0.05 was used). This analysis was performed using the software SigmaPlot version 11.0 (Systat Software, Inc., 2008).

## 3.3 Results and Discussion

### 3.3.1 Photocatalytic oxidation processes



There are several parameters that may influence the rate of photocatalytic degradation including pH, size and structure of the catalyst and oxidant and catalysts concentrations (Friedmann et al., 2010; Silva et al., 2007). The effect of pH on the photocatalytic degradation has been widely studied given that it can influence both, the surface charge proprieties of the catalyst, its propensity to form aggregates and the size of the aggregates and, the position of conductance and valence bands (Gaya and Abdullah, 2008; Rivas et al., 2001; Silva et al., 2007; Umar and Aziz, 2013). As shown in previous studies (Chatzisyneon et al., 2008; Ferreira et al., 2008; Gernjak et al., 2004; Kallel et al., 2009; Lucas and Peres, 2009; Ugurlu and Karaoglu, 2010), the optimum pH for the photodegradation of organic compounds (e.g. phenol) and in particular for the treatment of OMW seems to be between pH 2.0 and 5.0. Thus, in this study we decided not to adjust the pH of OMW, since after a 25% dilution it presented a pH of  $4.4 \pm 0.1$ . The variation of pH, also induces modifications in the ionized state of the surface of catalyst, which in turn depends on the point of zero charge (pH<sub>pzc</sub>). Therefore the surface of catalyst is positively (pH < pH<sub>pzc</sub>) or negatively (pH > pH<sub>pzc</sub>) charged depending if is in acidic or alkaline media, respectively (Chong et al., 2010; Gaya and Abdullah, 2008). After the addition of TiO<sub>2</sub> and Fe<sub>2</sub>O<sub>3</sub> NPs to the OMW solution the pH slightly increased (about 0.1) whereas in the systems with addition of H<sub>2</sub>O<sub>2</sub> (H<sub>2</sub>O<sub>2</sub>/UV and nano/H<sub>2</sub>O<sub>2</sub>/UV) the pH decreased. The effect of the amount of nano-TiO<sub>2</sub> and nano-Fe<sub>2</sub>O<sub>3</sub>, of the initial amount of H<sub>2</sub>O<sub>2</sub> in the UV/H<sub>2</sub>O<sub>2</sub> system and of the addition of H<sub>2</sub>O<sub>2</sub> into the photocatalyst system were investigated to determine the optimum operating conditions for removal of colour, aromatic compounds, COD and total phenolic content.

#### **3.3.1.1 Effect of photocatalysts and their concentrations**

Two NPs (TiO<sub>2</sub> and Fe<sub>2</sub>O<sub>3</sub>) were examined as semiconductors catalysts for the photocatalytic oxidation of OMW. One of the main factors affecting degradation studies is the amount of catalyst used. An excessive concentration of particles can affect light scattering and reduce the penetration of the light in the solution. So is important to determine the optimum dosage of the catalyst to ensure the total adsorption of photons (Ahmed et al., 2010; Chong et al., 2010). To determine the

optimal loading of catalyst, several amounts of nano-TiO<sub>2</sub> and nano-Fe<sub>2</sub>O<sub>3</sub> (0.25 to 2.0 g L<sup>-1</sup>) were added to OMW. Fig. 1 shows the results of different concentrations of nano-TiO<sub>2</sub> and nano-Fe<sub>2</sub>O<sub>3</sub> on the reduction (%) of colour, aromatic compounds, COD and total phenolic content after 2 hours of UV irradiation at effluent's inherent pH. The nano-assisted photocatalysis (UV/TiO<sub>2</sub> and UV/Fe<sub>2</sub>O<sub>3</sub>) resulted in reductions that do not go beyond 20% for colour, aromatic compounds, COD and total phenolic content except for the treatment with 0.75 g L<sup>-1</sup> of nano-TiO<sub>2</sub> with a reduction of 55 % for phenols ( $F = 8.517$ ; d.f. = 5,11;  $p = 0.002$ ) and for the 2.0 g L<sup>-1</sup> of nano-Fe<sub>2</sub>O<sub>3</sub> with a 23% reduction of COD ( $F = 9.396$ ; d.f. = 5,11;  $p=0.001$ ) (Fig.1). Similar results were obtained for COD (22% of reduction) by El Hajjouji et al. (2008) on a OMW sample treated with UV/TiO<sub>2</sub> (1 g L<sup>-1</sup>), but with a 100 fold effluent dilution and after 24h of treatment. Whereas in terms of coloration (at 330 nm) and phenols the same author's recorded reductions of 57% and 97%, respectively (El Hajjouji et al., 2008). We also recorded significant reductions for 1.0 g L<sup>-1</sup> and 2.0 g L<sup>-1</sup> of nano-TiO<sub>2</sub> in colour [for 1.0 g L<sup>-1</sup> ( $F = 140.570$ ; d.f. = 5,12;  $p < 0.001$ ), for 2.0 g L<sup>-1</sup> ( $F = 3.763$ ; d.f. = 5,11;  $p = 0.031$ )] and aromatic compounds [for 1.0 g L<sup>-1</sup> ( $F = 8.946$ ; d.f. = 5,12;  $p < 0.001$ ), for 2.0 g L<sup>-1</sup> ( $F = 9.777$ ; d.f. = 5,11;  $p < 0.001$ )]. In Fig. 1a we can see a relationship between the percentage of reduction for colour and the amount of nano-TiO<sub>2</sub>, when catalyst dosage increases from 0.25 to 2 g L<sup>-1</sup> and the same trend when the dosage increases from 0.25 to 1 g L<sup>-1</sup>, in terms of aromatic compounds reduction. In opposition for COD the percentage of reduction decreased when the concentration of nano-TiO<sub>2</sub> increased. However, when nano-Fe<sub>2</sub>O<sub>3</sub> was used as catalyst, a pronounced decrease in the removal efficiency of aromatic compounds was recorded (Fig. 1b). Other authors also report the same dependency on the catalyst dose (Badawy et al., 2009; Chatzisyneon et al., 2008; Mert et al., 2010). This means that although the increase in the number of active sites on catalyst surface for high concentrations of the catalyst, the penetration of UV light decreases due to shielding effect. Further, at high concentrations the aggregation of the catalysts can occur reducing the surface area of the particles and subsequently the number of the active surface sites (Ahmed et al., 2011; Nogueira et al., 2014). The Fig. 1b shows the increase in percentage of reduction of COD and total phenolic content with the increase of nano-Fe<sub>2</sub>O<sub>3</sub> concentration.

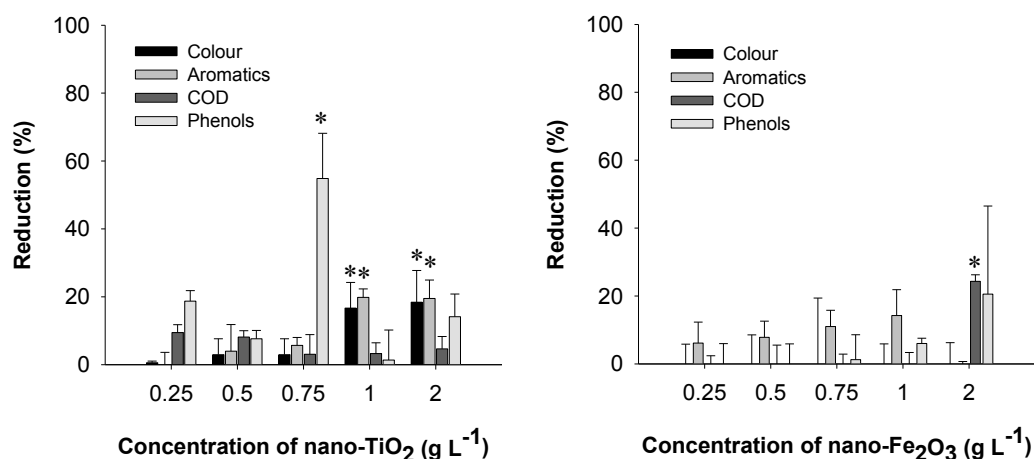


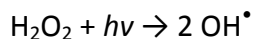
Figure III.1- Effect of nano-TiO<sub>2</sub> (a) and nano-Fe<sub>2</sub>O<sub>3</sub> (b) loadings on colour, aromatic compounds, COD and total phenolic content reduction after treatment of OMW at inherent pH and 2 hours of exposure to UV light. Asterisk stand for statistical significant differences from the control, for corresponding treatments (Dunnett test;  $p < 0.05$ ).

As can be seen in Fig. 1 (a and b), comparing photocatalysts effects, nano-TiO<sub>2</sub> seemed to be more effective than nano-Fe<sub>2</sub>O<sub>3</sub> in the reduction of almost all the parameters assessed. The observed results can be explained by the differences in particle size of the catalysts. Being the size of TiO<sub>2</sub> (< 25 nm) NPs smaller than the size of nano-Fe<sub>2</sub>O<sub>3</sub> ( $\approx 85 \times 425$  nm) NPs they will have a higher surface to volume ratio, and therefore more active sites will be available. Since all the reactions occur in the surface of the particles the efficiency to degrade organic compounds was higher for TiO<sub>2</sub>, as shown by the parameters evaluated, which was in accordance with the expectations (Gaya and Abdullah, 2008; Kwon et al., 2008).

### 3.3.1.2 Effect of hydrogen peroxide concentration in the H<sub>2</sub>O<sub>2</sub>/UV system

Hydrogen peroxide is known to be a strong oxidant and the direct photolysis of H<sub>2</sub>O<sub>2</sub> by UV light is widely used as an advanced oxidation process in the degradation of several organic compounds, such as phenols (Alnaizy and Akgerman, 2000; Esplugas et al., 2002; Ghaly et al., 2001; Pera-Titus et al., 2004; Stasinakis, 2008). The H<sub>2</sub>O<sub>2</sub>/UV

photolysis uses ultraviolet radiation (UV-B) to cleave hydrogen peroxide and generate  $\text{OH}^\bullet$  radicals:



The hydroxyl radicals are highly reactive and can attack organic compounds with low selectivity. Although this reaction may occur in nature itself, it is known that the toxicity of  $\text{H}_2\text{O}_2$  is concentration dependent causing harmful effects to several organisms and, when high concentrations are used. Hence, it stills necessary to develop better ways to remove it from the environment (Schmidt et al., 2006; Silva et al., 2007).

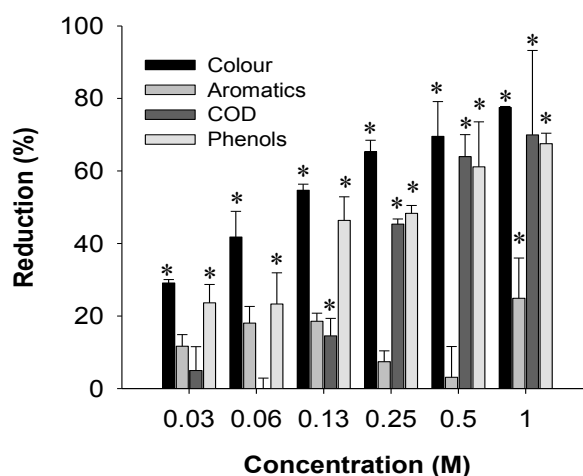


Figure III.2- Effect of  $\text{H}_2\text{O}_2$  loadings using  $\text{H}_2\text{O}_2/\text{UV}$  process on colour, aromatic compounds, COD and total phenolic content reduction after treatment of OMW at inherent pH and 2 hours of exposure to UV light. Asterisk stand for statistical significant differences from the control, for corresponding treatments (Dunnett tests;  $p < 0.05$ ).

Figure 2 shows the effect of  $\text{H}_2\text{O}_2$  concentration in the system  $\text{UV}/\text{H}_2\text{O}_2$  ranging from 0.03 to 1.00 M  $\text{H}_2\text{O}_2$  in the reduction of colour, aromatic compounds, COD and total phenolic content. It is notable that the percentage of reduction increases, in almost all the parameters tested, with increasing  $\text{H}_2\text{O}_2$  concentration. At the highest concentration of  $\text{H}_2\text{O}_2$  tested we reached percentages of reduction of 78%, 25%, 70% and 68% for colour, aromatic compounds, COD and total phenolic content, respectively. And only for this highest concentration of  $\text{H}_2\text{O}_2$  (1.00 M) significant differences were recorded for all the parameters (colour:  $F = 140.570$ ; d.f. = 5,12;  $p < 0.001$ , aromatics:  $F = 8.946$ ; d.f. = 5,12;  $p < 0.001$ , COD:  $F = 309.608$ ; d.f. = 5,10;  $p < 0.001$ ).

0.001, phenols:  $F = 148.844$ ; d.f. = 5,12;  $p < 0.001$ ), in comparison with the non-treated effluent. Uğurlu and Kula (Uğurlu and Kula, 2007) also tested the treatability of OMW by H<sub>2</sub>O<sub>2</sub>/UV treatment. After 7 days of treatment, using a H<sub>2</sub>O<sub>2</sub>/OMW ratio of 3 ml/100 ml, 90% of colour and phenol removal was achieved (Uğurlu and Kula, 2007). In another study Ahmed et al. (Ahmed et al., 2011) also applied the H<sub>2</sub>O<sub>2</sub>/UV system to treat a wastewater from olive oil milling industry and after 3h of treatment recorded removals of 75% and 65% for UV<sub>400</sub> and COD, respectively.

### 3.3.1.3 Effect of the addition of hydrogen peroxide on the photocatalytic system

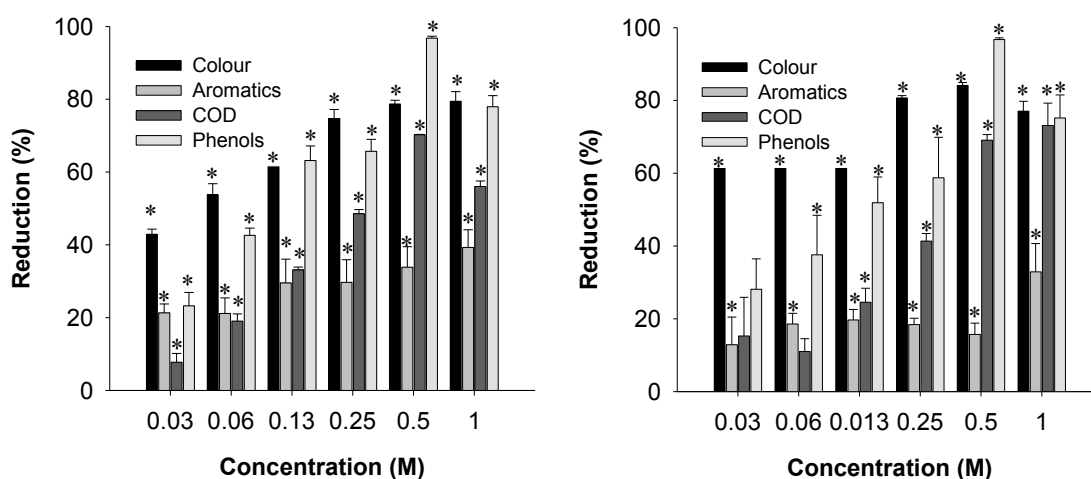
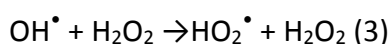
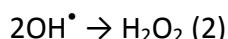
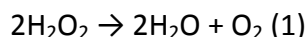


Figure III.3- Effect of H<sub>2</sub>O<sub>2</sub> loading in the process nano-TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub>/UV (a) and nano-Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub>/UV (b) on colour, aromatic compounds, COD and total phenolic content reduction at inherent pH, 1.0 g L<sup>-1</sup> catalyst loading and 2 hours of exposure to UV light. Asterisk stand for statistical significant differences from the control, for corresponding treatments (Dunnett tests;  $p < 0.05$ ).

Several experiments were carried out adding increasing concentrations of H<sub>2</sub>O<sub>2</sub> while catalyst dose (1.0 g L<sup>-1</sup>) was maintained constant. Although for some parameters the best reductions were recorded at a concentration of 2.0 g L<sup>-1</sup> (see Fig 1), we chose to use a catalyst loading of 1.0 g L<sup>-1</sup>, to reduce the possible of excess of catalyst, with higher costs involved, and as suggested by Chatzisyneon et al. (Chatzisyneon et al., 2008), to not mask the effect of other parameters involved in the photocatalytic

degradation (e.g. hydrogen peroxide). Figure 3 shows the effect of hydrogen peroxide addition on colour, aromatics, COD and total phenolic content reduction after 2 h of UV light, at a concentration of  $1.0 \text{ g L}^{-1}$  of catalyst. As can be seen in Fig. 3 (a and b) the addition of  $\text{H}_2\text{O}_2$  on the photocatalytic processes (nano- $\text{TiO}_2/\text{UV}$  and nano- $\text{Fe}_2\text{O}_3/\text{UV}$ ) enhanced the percentages of reduction for all the parameters assessed. For nano- $\text{TiO}_2/\text{H}_2\text{O}_2/\text{UV}$  system we reached maximum reductions of 79.4% for colour (with 1.00 M of  $\text{H}_2\text{O}_2$ ,  $F = 140.570$ ; d.f. = 5,12;  $p < 0.001$ ), 39.2% for aromatics (with 1.00 M of  $\text{H}_2\text{O}_2$ ,  $F = 8.946$ ; d.f. = 5,12;  $p < 0.001$ ), 70.3% for COD (with 0.50 M of  $\text{H}_2\text{O}_2$ ,  $F = 1034.326$ ; d.f. = 5,12;  $p < 0.001$ ) and 96.8% for phenols (with 0.50 M of  $\text{H}_2\text{O}_2$ ,  $F = 347.319$ ; d.f. = 4,10;  $p < 0.001$ ), while in the system nano- $\text{Fe}_2\text{O}_3/\text{H}_2\text{O}_2/\text{UV}$  we recorded reductions of 84.1% (with 0.50 M of  $\text{H}_2\text{O}_2$ ,  $F = 280.394$ ; d.f. = 4,10;  $p < 0.001$ ), 32.8% (with 1.00 M of  $\text{H}_2\text{O}_2$ ,  $F = 8.946$  d.f. = 5,12;  $p < 0.001$ ), 73.1% (with 1.00 M of  $\text{H}_2\text{O}_2$ ,  $F = 309.608$ ; d.f. = 5,10;  $p < 0.001$ ) and 96.7% (with 0.50 M of  $\text{H}_2\text{O}_2$ ,  $F = 38.445$ ; d.f. = 4,10;  $p < 0.001$ ). Increasing  $\text{H}_2\text{O}_2$  concentration improved the percentages of reduction. These results can be attributed to the possible formation of higher quantities of hydroxyl radicals since both  $\text{H}_2\text{O}_2$  and the catalyst contribute for the formation of oxidizing species (Ahmed et al., 2010). However, for COD and phenolic content, in the system  $\text{TiO}_2/\text{H}_2\text{O}_2/\text{UV}$  the maximum percentage of reduction was obtained at 0.50 M of  $\text{H}_2\text{O}_2$ , but above this  $\text{H}_2\text{O}_2$  concentration the percentage of reduction decreased. The same occurred for colour and phenolic content in the  $\text{Fe}_2\text{O}_3/\text{H}_2\text{O}_2/\text{UV}$  system. Although we cannot have a direct comparison between works, since the characteristics of the effluents and some of the tested parameters were different, several authors have reported the same effect, of a decreasing performance of  $\text{H}_2\text{O}_2$  with concentration increase (Badawy et al., 2009; Kallel et al., 2009; Mert et al., 2010). One of the reasons might be due to auto decomposition of  $\text{H}_2\text{O}_2$  into oxygen and water and the recombination of hydroxyl radicals as described by Eq. 1 and Eq. 2:



The excess of  $\text{H}_2\text{O}_2$  can itself react with  $\text{OH}^\bullet$  radicals (Eq. 3) or be adsorbed at the surface of catalyst competing with the organic compounds and consequently reducing

the efficiency of the treatment (Neyens and Baeyens, 2003). Further it is important to determine the optimum concentration of H<sub>2</sub>O<sub>2</sub> since residual hydrogen peroxide contributes to COD giving false positives (Kang et al., 1999; Lee et al., 2011; Talinli and Anderson, 1992) and can increase the toxicity of the treated wastewaters for several organisms (Gaikowski et al., 1999; Schmidt et al., 2006; Silva et al., 2007; Twiner et al., 2001). Several authors have been reporting that among the AOPs Fenton process is the most promising for the treatment of OMW, in this study we applied a Fenton-like process using nano-Fe<sub>2</sub>O<sub>3</sub> as well a photocatalysis process with nano-TiO<sub>2</sub>. At the best conditions of oxidant and catalyst in the photo-Fenton like process we recorded removal percentages of 84% for colour, 32% for aromatic compounds, 73% for COD and 96% for total phenolic content. Similar results were recorded when applied photo-Fenton oxidation to OMW samples. Justino et al. (Justino et al., 2009) recorded after four days of photo-Fenton treatment reduction percentages of 91%, 53% and 81% for colour, COD and total phenolic content without any dilution of the effluent. In a recent study, Papaphilippou et al. (Papaphilippou et al., 2013), tested the treatability of a raw OMW diluted 30 times (COD<sub>initial</sub>=1.950 g L<sup>-1</sup>) with photo-Fenton oxidation. After 240 min at pH=3 recorded reduction of 62% in terms of colour, 86% for COD and 83% for total phenols. In another study Ahmed et al. (Ahmed et al., 2011) applied photo-Fenton process to treat an actual agro-industrial wastewater from a olive oil milling industry. With an initial COD of 2000 mg O<sub>2</sub> L<sup>-1</sup> and after 3h of treatment they reached reduction of 95% for colour and 82% for COD. These studies showed that the use of the photo-Fenton process to treat this agro-industrial effluent is highly promising. However, the removal efficiencies depend on several factors such as COD initial concentration or effluent dilution, time of treatment, and, as mentioned before, on the concentration of catalyst and oxidant. In our study we used an effluent with a high value of COD ( $\approx 8.0$  g L<sup>-1</sup>), so comparing our results with the results reported by Ahmed et al. (Ahmed et al., 2011) and Papaphilippou et al. (Papaphilippou et al., 2013), the use of NPs on the photocatalytic process can greatly increase the percentages of reduction of the parameters tested. In the study of Justino et al. (Justino et al., 2009), no previous effluent dilution was made (COD<sub>initial</sub> $\approx 44.6$  g L<sup>-1</sup>) but the photo-Fenton process took 4 days while in our study only 2h of treatment were applied.

### 3.3.2 Toxicity to *Vibrio fischeri*

According to several authors, despite the great efficiency of AOPs in providing complete oxidation of the organic compounds, the possible formation of secondary intermediates can turn out the wastewater more toxic than the initial effluent without treatment. However most of the photo degradation studies did not include a toxicity evaluation (Justino et al., 2012; Rizzo, 2011). In this context the acute toxicity of OMW before and after the treatment was assessed using the marine bacteria *V. fischeri* and the results are showed on Table 1.

Table III.1- Values of EC<sub>50</sub> for bioluminescence inhibition expressed in effluent percentage, and corresponding 95% confidence intervals (CI95), for OOMW samples after the treatments at 5, 15, and 30 min exposure of *Vibrio fischeri* (NC not calculated).

	EC <sub>50</sub> (%)		
	Exposure time (min)		
	5	15	30
<b>Control</b>	<b>1.17% (CI95: 0.76-1.79)</b>	<b>1.25% (CI95: 0.79-1.99)</b>	<b>1.38% (CI95: 0.87-2.17)</b>
<b>UV/TiO<sub>2</sub> (0.25 g L<sup>-1</sup>)</b>	1.29% (CI95: 0.84-2.04)	1.32% (CI95: 0.86-2.07)	1.43% (CI95: 0.96-2.20)
<b>UV/TiO<sub>2</sub> (0.50 g L<sup>-1</sup>)</b>	1.14% (CI95: 0.68-1.90)	1.36% (CI95: 0.84-2.18)	1.92% (CI95: 1.23-2.99)
<b>UV/TiO<sub>2</sub> (0.75 g L<sup>-1</sup>)</b>	1.20% (CI95: 0.85-1.69)	1.23% (CI95: 0.87-1.74)	1.37% (CI95: 0.93-2.01)
<b>UV/TiO<sub>2</sub> (1.00 g L<sup>-1</sup>)</b>	1.10% (CI95: 0.56-1.11)	1.15% (CI95: 0.53-1.36)	1.13% (CI95: 0.52-1.27)
<b>UV/TiO<sub>2</sub> (2.00 g L<sup>-1</sup>)</b>	1.11% (CI95: 0.70-1.75)	1.25% (CI95: 0.78-1.99)	1.54% (CI95: 1.13-2.10)
<b>UV/Fe<sub>2</sub>O<sub>3</sub> (0.25 g L<sup>-1</sup>)</b>	1.25% (CI95: 0.78-2.00)	1.35% (CI95: 0.88-2.09)	1.41% (CI95: 0.89-2.26)
<b>UV/Fe<sub>2</sub>O<sub>3</sub> (0.50 g L<sup>-1</sup>)</b>	2.10% (CI95: 1.60-2.75)	2.12% (CI95: 1.56-2.90)	2.38% (CI95: 1.88-3.02)
<b>UV/Fe<sub>2</sub>O<sub>3</sub> (0.75 g L<sup>-1</sup>)</b>	1.35% (CI95: 0.88-2.07)	1.51% (CI95: 1.08-2.11)	1.55% (CI95: 1.08-2.23)
<b>UV/Fe<sub>2</sub>O<sub>3</sub> (1.00 g L<sup>-1</sup>)</b>	1.40% (CI95: 0.97-1.74)	1.51% (CI95: 1.05-2.00)	1.60% (CI95: 1.13-2.17)
<b>UV/Fe<sub>2</sub>O<sub>3</sub> (2.00 g L<sup>-1</sup>)</b>	1.29% (CI95: 0.80-2.11)	1.32% (CI95: 0.84-2.11)	1.35% (CI95: 0.86-2.17)
<b>UV/H<sub>2</sub>O<sub>2</sub> (0.03 M)</b>	1.56% (CI95: 1.18-2.06)	1.49% (CI95: 1.08-2.07)	1.29% (CI95: 0.84-1.98)
<b>UV/H<sub>2</sub>O<sub>2</sub> (0.06 M)</b>	2.54% (CI95: 2.21-2.94)	1.96% (CI95: 1.55-2.49)	1.70% (CI95: 1.43-2.03)
<b>UV/H<sub>2</sub>O<sub>2</sub> (0.13 M)</b>	3.34% (CI95: 2.62-4.29)	2.35% (CI95: 1.82-3.06)	1.83% (CI95: 1.34-4.99)
<b>UV/H<sub>2</sub>O<sub>2</sub> (0.25 M)</b>	4.32% (CI95: 3.27-5.70)	2.36% (CI95: 1.84-5.70)	2.40% (CI95: 1.91-3.00)
<b>UV/H<sub>2</sub>O<sub>2</sub> (0.50 M)</b>	21.49% (CI95: NC)	22.34% (CI95: NC)	21.95% (CI95: NC)
<b>UV/H<sub>2</sub>O<sub>2</sub> (1.00 M)</b>	41.69% (CI95: NC)	27.75% (CI95: NC)	13.34% (CI95: NC)
<b>UV/TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub> (0.03 M)</b>	1.54% (CI95: 1.29-1.84)	1.44% (CI95: 1.11-1.87)	1.37% (CI95: 0.98-1.90)
<b>UV/TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub> (0.06 M)</b>	3.09% (CI95: 2.14-4.48)	2.63% (CI95: 1.87-3.75)	2.23% (CI95: 1.55-3.22)
<b>UV/TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub> (0.13 M)</b>	4.43% (CI95: 2.90-7.10)	4.41% (CI95: 3.25-6.01)	3.60% (CI95: 2.74-4.73)
<b>UV/TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub> (0.25 M)</b>	4.03% (CI95: 2.38-7.07)	2.23% (CI95: 1.17-4.46)	1.41% (CI95: 1.39-2.04)



<b>UV/TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub> (0.50 M)</b>	9.58% (CI95: 5.41-17.12)	6.65% (CI95: 3.76-11.94)	5.02% (CI95: 2.83-9.23)
<b>UV/TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub> (1.00 M)</b>	6.45% (CI95: 4.64-8.17)	3.52% (CI95: 3.04-4.93)	3.47% (CI95: 2.86-6.30)
<b>UV/Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub> (0.03 M)</b>	1.21% (CI95: 0.90-1.62)	1.16% (CI95: 0.87-1.55)	1.18% (CI95: 0.84-1.65)
<b>UV/Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub> (0.06 M)</b>	1.80% (CI95: 1.18-2.76)	1.46% (CI95: 1.04-2.05)	1.26% (CI95: 0.98-1.61)
<b>UV/Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub> (0.13 M)</b>	2.67% (CI95: 2.25-3.14)	2.16% (CI95: 1.85-2.53)	2.68% (CI95: 2.26-3.17)
<b>UV/Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub> (0.25 M)</b>	5.90% (CI95: 4.97-7.01)	4.63% (CI95: 3.91-5.48)	4.54% (CI95: 3.80-5.43)
<b>UV/Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub> (0.50 M)</b>	7.23% (CI95: 4.54-11.87)	5.28% (CI95: 3.47-8.01)	4.16% (CI95: 2.87-6.13)
<b>UV/Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub> (1.00 M)</b>	15.45% (CI95: NC)	14.34% (CI95: NC)	16.87% (CI95: NC)

The non-treated effluent (control) was highly toxic to *V. fischeri* since almost completely inhibited the bioluminescence of the bacteria presenting extremely low EC<sub>50</sub> values never exceeding 1.38 % (Table 1). Also the toxicity of nano-TiO<sub>2</sub> and nano-Fe<sub>2</sub>O<sub>3</sub> suspensions was assessed, at the highest concentrations used in the treatments (1.0 g L<sup>-1</sup>) and for both NPs no inhibitory effects were recorded. In the treatments using only the catalyst (TiO<sub>2</sub>/UV and Fe<sub>2</sub>O<sub>3</sub>/UV) the toxicity to *V. fischeri* remained practically the same, only for Fe<sub>2</sub>O<sub>3</sub>/UV (0.50 g L<sup>-1</sup>) a slight decrease occurred (EC<sub>50</sub> = 2.38% at 30 min). The best reduction in terms of toxicity was achieved in the treatments with only the oxidant (H<sub>2</sub>O<sub>2</sub>/UV) (Table 1) after 5 min of exposure (e.g EC<sub>50</sub> of 41.69%), although with increasing exposure time the toxicity increased. In terms of efficiency, the systems associating nano-TiO<sub>2</sub> or nano-Fe<sub>2</sub>O<sub>3</sub> with H<sub>2</sub>O<sub>2</sub> were the best processes; however the reduction of toxicity was not as high as in the treatment with only the oxidant. Several authors reported that although this type of processes, based on photo-Fenton like reaction, are capable of high reductions in COD, phenolic's content or colour, usually this is not followed by a decrease in terms of toxicity (Ferreira et al., 2008; Justino et al., 2009; Papaphilippou et al., 2013; Pereira et al., 2009). These observations are in agreement with this study. In some cases the toxicity after the oxidation process even increase, as reported by Papaphilippou et al. (Papaphilippou et al., 2013). They performed phytotoxicity tests with three plant species (*Sorghum saccharatum*, *Lepidium sativum* and *Sinapis alba*) and toxicity assays with *D. magna* (immobilization), before and after photo-Fenton oxidation, and for both assays the author's confirmed that the oxidized samples were always more toxic than the raw effluent. The formation of more toxic by-products due to the partial oxidation

of organic compounds was the explanation suggested for the increased toxicity (Papaphilippou et al., 2013). The same interpretation was made by Pereira et al. (Pereira et al., 2009) after the treatment of bleached kraft pulp mill effluent with photo-Fenton process. In summary, the increased toxicity over time recorded for some NPs/H<sub>2</sub>O<sub>2</sub>/UV treatments was probably due to the incomplete oxidation of organic contaminants with the formation of more toxic intermediated compounds (Oller et al., 2011; Rizzo, 2011).

### 3.4 Conclusion

The treatment of a highly toxic agroindustrial effluent like the OMW is of great importance. It is imperative to develop treatments capable of reducing the environmental impact of OMW discharges. Our results show that the treatment of olive oil mill wastewater using advanced oxidation processes is appropriate for removing colour, aromatic compounds, COD and total phenolic content. The general efficiency is dependent of operation conditions such as catalyst type and concentration, and the use of an oxidant. The use of nano-TiO<sub>2</sub> or nano-Fe<sub>2</sub>O<sub>3</sub> and H<sub>2</sub>O<sub>2</sub> combined greatly enhance the efficiency of the photocatalytic process for the physico-chemical parameters. The efficiency of the treatments decreased as following: UV/TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub> and UV/Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub> > UV/H<sub>2</sub>O<sub>2</sub> > UV/TiO<sub>2</sub> > UV/Fe<sub>2</sub>O<sub>3</sub>. In terms of reducing toxicity to the bacteria *Vibrio fischeri* the most promising treatment was UV/H<sub>2</sub>O<sub>2</sub> system. Further work should be performed in order to optimize all the conditions for a possible industrial application. Although several studies reporting the different advantages of nanoparticles in remediation and wastewater treatment abound in the literature, we showed with our results that many evaluations have to be made before the up-scaling of these approaches, and it will also be important, in future studies, to access the fate and potential impacts of solid wastes formed and released after wastewater treatment with nanoparticles. Overall, if we assume a compromise between the improvement of physical and chemical parameters and of toxicity of the resulted OMW, the best combination was achieved using nano-Fe<sub>2</sub>O<sub>3</sub> with hydrogen peroxide and UV light.

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# Chapter IV

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**Biological treatment with fungi of olive oil mill wastewater pre-treated by photocatalytic oxidation with nanomaterials.**



## Biological treatment with fungi of olive oil mill wastewater pre-treated by photocatalytic oxidation with nanomaterials

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### Abstract

Olive oil mill wastewaters (OOMW) still a major environmental problem due to the high chemical oxygen demand (COD) and total phenolic content (TPC), which are usually toxic and recalcitrant. Several attempts have been made in developing more efficient treatment processes, but no chemical or biological treatments were found to be totally effective, especially in terms of toxicity reduction. The main purpose of this study was to investigate the treatability of OOMW by the combination of photocatalytic oxidation, using two nanomaterials as catalysts ( $\text{TiO}_2$  and  $\text{Fe}_2\text{O}_3$ ), with biological degradation by fungi (*Pleurotus sajor caju* and *Phanerochaete chrysosporium*). Photocatalytic oxidation was carried out using different systems, nano- $\text{TiO}_2$ /UV, nano- $\text{Fe}_2\text{O}_3$ /UV, nano- $\text{TiO}_2$ / $\text{H}_2\text{O}_2$ /UV and nano- $\text{Fe}_2\text{O}_3$ / $\text{H}_2\text{O}_2$ /UV. The effectiveness of the treatment was assessed through colour (465 nm), aromatics (270 nm), COD and TPC reductions, as well decrease of toxicity using the bacterium *Vibrio fischeri*. The chemical treatment alone using the system nano- $\text{TiO}_2$ / $\text{H}_2\text{O}_2$ /UV promoted 43%, 14%, 38% and 31% reductions for colour, aromatic content, COD and phenolic content, respectively. However no toxicity reduction was observed. The combination with a biological treatment increased the reduction of COD and TPC as well promoted toxicity reduction. The treatment with *P. chrysosporium* promoted the higher reductions in terms of toxicity, but the *P. sajor caju* promote the best reductions of COD and TPC. However, the biological treatment was more effective when no hydrogen peroxide was used in the pre-treatment.

**Keywords:** Olive oil mill wastewater, biological treatment, fungi, photocatalytic oxidation, nanomaterials

## 4.1 Introduction

Olive oil production is an important economic activity in the Mediterranean countries. However, this agro-food industry produces a meaningful negative externality, corresponding to large volumes of a wastewater particularly difficult to treat, which is responsible for serious environmental contamination problems. Olive oil mill wastewater (OOMW) is characterized by a foul-smelling, dark colour, acid pH and high organic content, mainly composed by sugars, tannins, pectins, polyphenols, polyalcohols and lipids (Dermeche et al. 2013). The high levels of chemical and biological oxygen demand (COD and BOD levels) and the high content in recalcitrant phenolic compounds are responsible for the lethal, phytotoxic and microbial growth inhibition effects of the OOMW (Asfi et al. 2012; Justino et al. 2009). However, despite of the awareness of the toxic potential of this effluent it continues to be spread without proper treatment (Justino et al. 2012). It is then of crucial importance to find effective treatments to treat OOMW before its safe disposal in the environment.

Thereby, several methods were already tested, including physical, chemical and biological processes, aimed in promoting the degradation of the complex organic load of this effluent (Kilic and Solmaz 2013; McNamara et al. 2008; Paraskeva and Diamadopoulos 2006). None of these single methods have proved to be completely efficient for OOMW. Biological processes are within the most environmental friendly (no secondary sludge are produced) and economic treatments, however are ineffective for OOMW due to the high level of antimicrobial compounds (Hanafi et al. 2010). Moreover, high dilution percentages are usually required before biological treatments (Justino et al. 2012; Mantzavinos and Kalogerakis 2005). White-rot fungi (e.g. *Phanerochaete spp.* and *Pleurotus spp.*) have been pointed out as the most suitable organisms to treat OOMW due to their capacity for degrading complex compounds like phenols which have a lignin-like structure (Morillo et al. 2009; Yesilada et al. 1999). Through their extracellular ligninolytic enzymes, such as laccase, manganese peroxidase and lignin peroxidase, they are able to catalyse the degradation of lignin and the oxidation of persistent aromatic and halogenated compounds (Aytar et al. 2013; Baldrian 2006). To overcome the limitations caused by the toxicity of OOMW to biota, a dilution step or a pre-treatment may be required before the contact with fungi. The treatment of wastewaters combining chemical and biological processes has

been reviewed in the literature (Mantzavinos and Kalogerakis 2005; Scott and Ollis 1995). Some of the most commonly applied and promising technologies for OOMW are the advanced oxidation processes (AOPs), which were reported as effective in removing recalcitrant organic compounds, thus improving the biodegradability of this wastewater (Badawy et al. 2009; Michael et al. 2014; Stasinakis 2008). These processes are based on the in situ formation of highly reactive and oxidizing species, such as hydroxyl radicals, which accelerate the oxidation of a vast array of organic and inorganic pollutants with relatively low selectivity (Kestioğlu et al. 2005; Rizzo 2011). Most of these methods consist in combinations of oxidizing agents (e.g.  $\text{H}_2\text{O}_2$ ,  $\text{O}_3$ ), ultraviolet or solar irradiation and catalysts (e.g.  $\text{TiO}_2$ ,  $\text{Fe}_2\text{O}_3$ ,  $\text{ZnO}$ ). Among the different methods available, the Fenton's like reactions and heterogeneous photocatalysis with titanium dioxide, showed a great potential for the pre-treatment of organic wastewaters (Jamil et al. 2011; Justino et al. 2010). Badawy et al. (2009) compared both photo-Fenton and  $\text{TiO}_2$ /UV processes as a pre-treatment step to enhance the biodegradability of OOMW. The efficiency of both processes in terms of COD, TOC (total organic carbon), total phenolic compounds and total suspended solids (TSSs) was shown by the removal percentages of 87%, 84%, 97.44% and 98.31% attained for the four parameters, respectively, with photo-Fenton treatment and, of 68.8%, 67.3%, 40.2% and 48.9%, respectively, for the  $\text{TiO}_2$ /UV treatment. In parallel, the  $\text{BOD}_5/\text{COD}$  ( $\text{BOD}_5$  – biochemical oxygen demand after 5 days) ratio increased for both treatments confirming the ability of these processes to enhance the biodegradability of the recalcitrant compounds of OOMW (Badawy et al. 2009).

In the last few years nanoparticles (NPs) attracted lots of attention, since an improvement in the oxidation process is expected when they are applied as catalysts. In fact, decreasing the particle size, the band gap energy and the surface to volume ratio of the NPs increase greatly, leading to more active surfaces for adsorption and also a higher redox potential (Khin et al. 2012; Theron et al. 2008).

The oxidation processes have shown great efficiency in the complete or almost complete mineralization of several recalcitrant organic compounds from the OOMW. However, the complete mineralization is highly expensive (Machulek Jr. et al. 2013; Oller et al. 2011). Thus the combination of chemical and biological processes is gaining a great interest for the treatment of wastewaters with high and complex organic

contents (Badawy et al. 2009). And the nanomaterials can greatly enhance the chemical pre-treatments (Han and Bai 2009; Swetha et al. 2010).

Thus the main aim of the present study was to assess the combination of photocatalytic (nano-TiO<sub>2</sub>/UV, nano-Fe<sub>2</sub>O<sub>3</sub>/UV, nano-TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub>/UV and nano-Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub>/UV) and biological (*P. chrysosporium* and *P. sajor caju*) treatments, in improving the chemical characteristics of an OOMW and in reducing its toxicity. The chemical pre-treatment was applied to a lower dilution of the OOMW (50%) when compared with those applied on previous published studies (Ntougias et al. 2012; Jarbouy et al. 2013). The efficiency of combined treatments was assessed through aromatics (270nm), colour reduction (465nm), COD, TPC and ecotoxicity (Microtox®) reduction. To best of our knowledge, this is the first study applying biological treatment with fungi of olive mill wastewater pre-treated by photocatalytic oxidation with nanomaterials.

## **4.2 Materials and Methods**

### **4.2.1 Olive oil mill wastewater source**

Olive oil mill wastewater (OOMW) was collected from an evaporation pond immediately after the annual olive oil production and was the by-product of a three-phase olive oil extraction process. OOMW was stored in screw-caped glass flasks, at 4°C, until the start of the experiments. Before use OOMW was grossly filtered with a filter paper to remove the coarse particles under suspension and previously 50% diluted with distilled water. No further dilutions were made for the chemical pre-treatment or the biological process. The major characteristics of the diluted effluent were: COD= 16.5±0.6 g L<sup>-1</sup>, TPC= 135.8±7.2 mg L<sup>-1</sup> and pH 4.5±0.1 (Table 1).

### **4.2.2. Nano-catalysts and oxidant reagent**

The two NMs were purchased as nanopowders. The titanium (IV) oxide anatase (TiO<sub>2</sub>) was purchased from Sigma Aldrich, with particle size < 25nm and 99.7% metal basis. The iron (III) oxide nanorods (Fe<sub>2</sub>O<sub>3</sub>) had an average particle size of 85x425 nm (d=40-130nm, l=250-600nm) and purity above 99% and, were purchased from Nanostructured & Amorphous Materials Inc. (Houston, Texas, USA). Hydrogen peroxide (30%, analytical grade) was purchased from Fisher Scientific. The toxicity of



both NMs was previously tested for a battery of aquatic species (both freshwater and marine species) for concentrations up to  $20 \text{ mg L}^{-1}$ , as well for concentrations up to  $2 \text{ g L}^{-1}$  to the bacteria *Vibrio fischeri* (Chapter II and III). Both NMs were chosen because they were less toxic in the range of concentrations tested.

#### 4.2.3 Photocatalytic degradation

Photodegradation treatments were carried out using 200 ml of OOMW in 250 ml glass beakers at room temperature ( $20^{\circ}\text{C}$ ). The treatment was made without pH adjustment because in the literature pHs between 2 and 5 were considered optimum for the photodegradation processes (Chatzisyneon et al. 2008; Ferreira et al. 2008; Kallel et al. 2009). Three replicates were prepared per treatment. The catalysts (nano- $\text{TiO}_2$  or nano- $\text{Fe}_2\text{O}_3$ ) were added to each beaker at a concentration of  $1.0 \text{ g L}^{-1}$ . The suspensions (effluent + catalyst) were placed on in orbital shaker (100 rpm) overnight to attain the adsorption–desorption equilibrium for the organic compounds on the catalyst surface. Silva et al. (2007) and Chatzisyneon et al. (2008) reported that for a initial concentration of  $\text{COD}=1.0 \text{ g L}^{-1}$  the adsorption equilibrium was achieved after more or less 30 min. After this period the UV lamp (Spectroline XX15F/B, Spectronics Corporation, NY, USA, peak emission at 312 nm) was switch on, and left for 2 hours. During irradiation, agitation was maintained by magnetic stirring to keep the suspensions homogenous.

According to the literature, the presence of strong oxidant species (e.g.  $\text{O}_3$ ,  $\text{H}_2\text{O}_2$ ) can greatly enhance the photodegradation process (Chiou et al. 2008; Jamil et al. 2011). This led us to test the systems nano- $\text{TiO}_2/\text{H}_2\text{O}_2/\text{UV}$  and nano- $\text{Fe}_2\text{O}_3/\text{H}_2\text{O}_2/\text{UV}$  by adding 0.50 M of  $\text{H}_2\text{O}_2$  before UV irradiation in the same conditions as stated above, in new replicates prepared for this purpose (three per treatment). The catalyst and oxidant concentrations were selected based on our previous study (Chapter III). The samples of treated effluent were left for 5 days, at room temperature, during which the oxidation reaction proceed with the release of carbon dioxide and the production of a precipitate. Aliquots (5 ml) of each replicate were withdrawn throughout oxidation period, after the 2h of UV as well as 2 and 5 days for absorbance monitoring (270 and 465 nm). After this period, the sub-samples were filtered through GF/F ( $\varnothing 47\text{mm}$ ,

Whatman) glass-fiber filter to remove the catalyst before analysis, for chemical characterization (COD and total phenolic content) and ecotoxicological evaluation.

In the treatments with  $\text{H}_2\text{O}_2$ , samples were withdrawn for analysis of COD and for the ecotoxicity tests with *V. fischeri* (herein described) after the addition of sodium hydroxide (0.6 N) and heating in a bath ( $100^\circ\text{C}$ ) for 4h. With this procedure we aimed to attain the complete elimination of residual hydrogen peroxide. It has been shown that the rate of  $\text{H}_2\text{O}_2$  decomposition (to oxygen and water) increases several folds as pH increases and temperature rises (Qiang et al. 2002).

#### 4.2.4 Biological treatment

The basidiomycete fungi species used in this study for the biological treatment of OOMW, namely *P. sajor caju* and *P. chrysosporium* (Burdsall 38388) were obtained from UNESP (São Paulo State University, Brazil) and from BCCMTM/MUCL Culture Collection (Belgium), respectively. *P. sajor caju* was cultured at  $25^\circ\text{C}$ , while *P. chrysosporium* was cultured at  $30^\circ\text{C}$ , in a medium containing  $20\text{ g L}^{-1}$  of malt extract,  $1\text{ g L}^{-1}$  of peptone and  $16\text{ g L}^{-1}$  of agar. After a growth period, all species were preserved at  $4^\circ\text{C}$  during 5 days, in the culture medium to maintain the mycelium hydrated and with nutrients.

Before the biological treatment of OOMW, mycelia of *P. sajor caju* and of *P. chrysosporium* were grown in a liquid medium containing  $20\text{ g L}^{-1}$  of malt extract and  $1\text{ g L}^{-1}$  of peptone, at  $25^\circ\text{C}$  and  $120\pm 10\text{ rpm}$ . After growth, the mycelia was collected filtering the culture medium, through a sterilized gaze and were kept in sterilized plastic containers at  $4^\circ\text{C}$ , until the biological treatment of OOMW.

The biological treatment of OOMW samples was performed by adding the ligninolytic fungi *P. sajor caju* and *P. chrysosporium* ( $5.0\pm 0.1\text{ g}$ ) individually to 190 ml of pre-diluted and treated OOMW by the photodegradation process (above described) in glass beakers. All the OOMW samples were filtered through GF/F ( $\varnothing 47\text{mm}$ , Whatman) glass-fiber filter to remove the catalyst particles prior to the addition of fungi biomass. The fungi biomass added to each beaker, was based on the study of Ferreira et al. (2008). According to Jaouani et al. (2003), the following additives were added to the OOMW samples for the biological treatment:  $1.0\text{ g L}^{-1}$  of potassium dihydrogen phosphate,  $0.405\text{ g L}^{-1}$  of diammonium tartrate dibasic and  $0.05\text{ g L}^{-1}$  of yeast extract.

Three replicates per photodegradation pre-treatment and fungus species, as well three replicas with no photodegradation pre-treatment, were inoculated with each fungus mycelia, in sterilized Erlenmeyer flasks covered up with sterile hydrophobic cotton and gauze, and incubated at 25°C, 120±10 rpm, for 21 days. Throughout the incubation period, sub-samples of 5 ml of the treated effluent were withdrawn from each treatment at different moments (0, 3, 7, 10, 14, 17, 21 days) in order to monitor pH and absorbance at 270 and 465 nm. Before the biological treatment and after 21 days of incubation with fungi, chemical characterization (COD and TPC) and toxicity assessment was also performed on OOMW samples.

#### 4.2.5 Post-treatment evaluations

The absorbance measurements before and after the chemical and biological treatments were performed using a UV/VIS spectrophotometer (Shimadzu 1800) at wavelengths associated with aromatic (270 nm) and colored (465 nm) compounds. The COD was analyzed following the standard method from the American Society for Testing Materials D 1252-88 (ASTM, 1994). The pH was measured with a pH meter (pH meter 330 from WTW). Total phenolic content was determined by the colorimetric method of Folin–Ciocalteu (Mulinacci et al. 2001).

After the withdrawal of samples for chemical characterization the biomass of *P. sajor caju* and *P. chrysosporium*, was harvested by filtering the content of each beaker through a filter paper. The dry weight of fungi was determined after drying at 60°C, until a constant weight is reached. Fungi biomass increments were assessed weighing dry biomass before and after each biological treatment. The acute toxicity of all the samples, before and after the pre-treatment and the biological treatment, was assessed following the 81.9% Basic Test protocol provided by the supplier, using a Microtox Model 500 Analyser (Azur Environmental 1998). The endpoint measured in the Microtox® assay was the decrease in the intensity of light emitted by the luminescent marine bacteria – *Vibrio fischeri* – after 30 min of exposure to several dilutions of each effluent sample (by using a dilution factor of 2x), after previous osmotic and pH (6-8) adjustment of the samples.

#### 4.2.6 Statistical analysis

The EC<sub>50</sub> values and corresponding 95% confidence intervals for *V. fischeri* were computed using the MicrotoxOmni® software version V1.18 with a linear model (Azur Environmental 1998). One-way ANOVAs followed by Dunnett tests were performed to find out potential significant differences between the control (non-treated effluent) and the chemical treated effluent samples. A two-way analysis of variance (ANOVA) was performed to test for the significant effect of both factors: the chemical pre-treatment and the fungi species used for the biological treatment. The effect of pre-chemical treatment and biological treatment period on colour, aromatic compounds, fungal growth, pH, COD, phenolic content and toxicity was also tested. When significant interactions ( $p < 0.05$ ) between both factors were observed, differences between groups were tested by simple main effects ANOVA followed by a multicomparison Tukey test. For the toxicity the raw data were transformed by using the arcsin transformation and for pH the Log<sub>10</sub> function, to meet ANOVA assumptions. All analyses were performed by means of Sigmaplot version 11.0 software program (Systat Software, Inc., 2008).

## **4.3 Results and Discussion**

### **4.3.1 Photochemical treatments**

The degradation of OOMW was evaluated by employing photocatalysis with nano-TiO<sub>2</sub> with and without the presence of H<sub>2</sub>O<sub>2</sub> (nano-TiO<sub>2</sub>/UV and nano-TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub>/UV), and through Fenton like reaction with nano-Fe<sub>2</sub>O<sub>3</sub> (nano-Fe<sub>2</sub>O<sub>3</sub>/UV and nano-Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub>/UV). Figure 1 shows the relative absorbance ratio  $[(Abs(t)/Abs(t_0))]$  for colored and aromatic compounds after 5 days of chemical oxidation with the different pre-treatments applied. In terms of colour reduction we only recorded significant differences between the control and the treatments using hydrogen peroxide in the system (nano-TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub>/UV and nano-Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub>/UV) with percentages of reduction of 43.5% and 48.0% ( $F_{4,10}=15.257$ ;  $p < 0.001$ ), respectively. However for aromatic compounds (absorbance measurements at 270 nm) no significant reduction was recorded for all the treatments ( $F_{4,10}=2.379$ ;  $p=0.121$ ). In fact a reduction was observed after 2 days of treatment, but the levels recovered for the initial ones, at the end. This increment in colour was probably due to the polymerization of aromatic compounds into polymers with high molecular mass

(Khoufi et al. 2006, 2004). In parallel with reductions in the absorbance ratio at 465 nm, significant reductions in COD ( $F_{4,10}=120.856$ ;  $p<0.001$ ) and TPC ( $F_{4,10}=23.258$ ;  $p<0.001$ ) were recorded only for the treatments with  $H_2O_2$ . We observed percentages of reduction of 38.0% and 36.9% for COD, and 31.2% and 25.5% for TPC for the treatments with nano- $TiO_2/H_2O_2/UV$  and nano- $Fe_2O_3/H_2O_2/UV$ , respectively (Table 1). Photodegradation with previous processes was much more effective than those without hydrogen peroxide. The addition of hydrogen peroxide to the process increased the formation of hydroxyl radicals, which are considered as the main responsible for the oxidative degradation. In these processes both the catalysts and  $H_2O_2$  contributed for the formation of radicals (Ahmed et al. 2010). In the study of Garcia et al. (2007) the treatability of a real textile effluent with  $TiO_2(P25, \text{size } 21 \text{ nm})/UV$ ,  $H_2O_2/UV$ ,  $TiO_2/H_2O_2/UV$  and non-nano  $Fe^{2+}/H_2O_2/UV$  was tested. Also for these authors the most effective treatments with the highest mineralization rates were the ones using  $TiO_2(P25)/H_2O_2/UV$  and non nano- $Fe^{2+}/H_2O_2/UV$  systems. Our results were also coincident with those from Primo et al. (2008). In this study several processes (non-nano  $Fe^{2+}/H_2O_2/UV$ , non-nano  $Cu^{2+}/H_2O_2$ , non-nano  $Fe^{2+}/H_2O_2$ ,  $H_2O_2/UV$  and  $UV$ ) were investigated to treat landfill leachates and it was found that photo-Fenton processes, combining catalyst and hydrogen peroxide in the presence of UV light, were the most efficient one, promoting reductions in COD of about 78%. Although the reduction in COD, recorded in this study from Primo et al (2008), with non nano-catalysts was higher than the reduction recorded in our study, the initial concentration of COD of the OOMW ( $16.50 \text{ g L}^{-1}$ , after 50% dilution) was about 4x higher than that of the landfill leachates ( $3.82 \text{ g L}^{-1}$ ) (Primo et al. 2008). Thus, we cannot assume immediately that nano catalysts are less efficient in the oxidation of organic compounds.

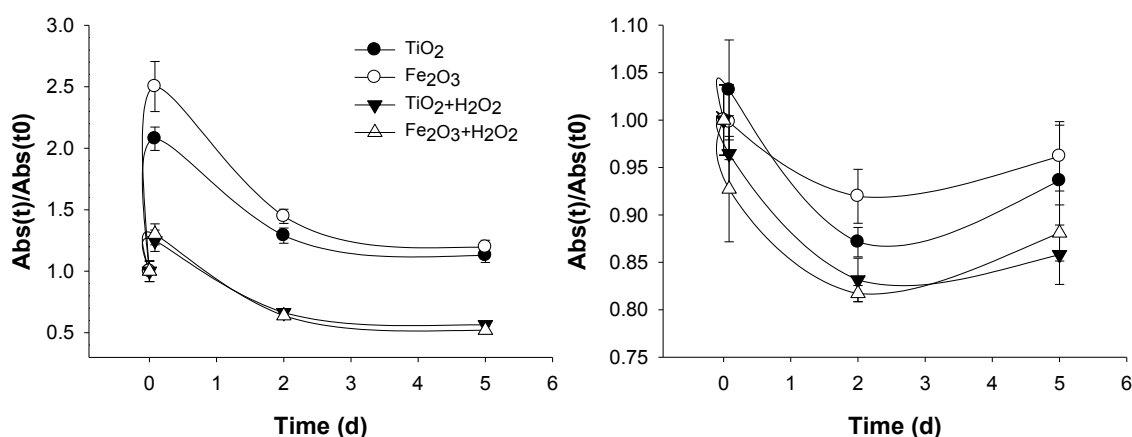


Figure IV.1- OMW absorbance ratio  $[Abs(t)/Abs(t_0)]$  at 465 nm (a) and 270 nm (b) after 5 days of chemical oxidation with nano- $TiO_2$ /UV ( $TiO_2$ ), nano- $Fe_2O_3$ /UV ( $Fe_2O_3$ ), nano- $TiO_2/H_2O_2$ /UV ( $TiO_2+H_2O_2$ ) and nano- $Fe_2O_3/H_2O_2$ /UV ( $Fe_2O_3+H_2O_2$ ).

To determine the efficiency of the treatments applied, in terms of toxicity reduction,  $EC_{50}$  values for the bacteria *V. fischeri* were obtained before and after each chemical pre-treatment. The raw effluent with a 50% dilution was highly toxic to *V. fischeri* recording an  $EC_{50-30min}$  value 1.82%. As it was possible to observe a slight decrease in toxicity occurred after the treatments nano- $TiO_2/H_2O_2$ /UV ( $EC_{50-30min}$  = 2.61%) and nano- $Fe_2O_3/H_2O_2$ /UV ( $EC_{50-30min}$  = 2.19%). However, the OOMW samples remained highly toxic. No statistical significant difference was recorded for the toxicity of all the treatments before and after the chemical pre-treatment (Table 1). The photocatalytic treatments have proved their efficiency in terms of COD, phenol and color reduction. However, for the great majority of the cases this improvement in the chemical properties of the effluent was not followed by a reduction in toxicity what is in agreement with other studies (Papaphilippou et al. 2013; Pereira et al. 2009). This increment or maintenance of the toxicity of the effluent, after the photocatalytic treatment was probably due to the formation of reaction by-products resulting from the partial oxidation of organic compounds, which sometimes are more toxic than their parent compounds (Rizzo 2011). Pereira et al. (2009) reported the same tendency, showing an increment in the toxicity to *V. fischeri*, *Raphidocelis subcapitata*, *D. magna* and *D. longispina* of a bleached kraft pulp mill effluent, after photo-Fenton treatment with non-nano catalysts applied for 6 days. Papaphilippou et al. (2013) evaluated the acute toxicity of an OOMW, before and after photo-Fenton oxidation,

for *D. magna* and three plants species (*Sorghum saccharatum*, *Lepidium sativum* and *Sinapis alba*). These authors also observed the same tendency, as the oxidized samples were always more toxic than the raw effluent for all the species tested. Nevertheless, in both studies such increment in toxicity could have been caused by residual  $H_2O_2$  in the treated samples, since no additional measures were taken to completely eliminate it, at the end of the treatment.

The pre-treatment that showed the best results, improving the physical and chemical parameters as well the toxicity of the OOMW used in this study was the nano-TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub>/UV system. The nano-TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub>/UV treatment promoted reductions of 43.5% in color, 14.2% in aromatics, 38.0% in COD, 31.2% in TPC and also promoted a slight decrease (although non-significant) in the toxicity of the effluent ( $EC_{50-30min}$  = 2.61%) (Table 1).

Table IV.1- Average  $\pm$  STDEV values of pH, COD, total phenolic content ( $mg\ L^{-1}$ ), % of reduction in COD and TPC and toxicity of OOMW samples treated by the four chemical treatments previously described. Toxicity data is expressed in % of dilution of the OOMW sample tested. Asterisk stand for statistical significant differences from the control, for corresponding treatments (Dunnett tests;  $p < 0.05$ ).

Chemical treatment	pH		COD ( $g\ L^{-1}$ )		Total phenolic content (TPC) ( $mg\ L^{-1}$ )		Reduction (%)		$EC_{50\ 30min}$ (CI95)
	0d	5d	0 d	5d	0d	5d	COD	TPC	
TiO <sub>2</sub> /UV	4.5 $\pm$ 0.1	4.3 $\pm$ 0.02	16.5 $\pm$ 0.6	15.8 $\pm$ 2.9	135.8 $\pm$ 7.2	132.5 $\pm$ 7.6	4.4	5.5	1.79% (1.25-2.56)
Fe <sub>2</sub> O <sub>3</sub> /UV	4.5 $\pm$ 0.1	4.4 $\pm$ 0.02	16.5 $\pm$ 0.6	18.1 $\pm$ 0.6	135.8 $\pm$ 7.3	120.7 $\pm$ 4.6	-9.5	9.9	1.67% (1.23-2.27)
TiO <sub>2</sub> /H <sub>2</sub> O <sub>2</sub> /UV	4.5 $\pm$ 0.1	4.4 $\pm$ 0.01	16.5 $\pm$ 0.6	10.2 $\pm$ 0.3*	135.8 $\pm$ 7.4	90.1 $\pm$ 9.0*	38.0	31.2	2.61% (2.12-3.23)
Fe <sub>2</sub> O <sub>3</sub> /H <sub>2</sub> O <sub>2</sub> /UV	4.5 $\pm$ 0.1	4.5 $\pm$ 0.01	16.5 $\pm$ 0.6	10.4 $\pm$ 0.4*	135.8 $\pm$ 7.5	105.0 $\pm$ 3.1*	36.9	25.5	2.19% (1.60-3.01)

#### 4.3.2 Biological treatment of the pre-treated OMW

In Fig. 2 it is possible to observe that great oscillations occurred in the absorbance ratio  $[(Abs(t)/Abs(t_0))]$  at 465 and 270 nm throughout the incubation period of pre-treated OOMW samples with both fungi species used in this study. The 21 days of biological treatment did not decrease the effluent colour. In opposition at the end of the treatment with *P. chrysosporium* the absorbance ratio reached extremely high values at 465 nm (Fig. 2). Only after 3 days of biological treatment with

*P. chrysosporium* a significant reduction in colour was recorded for the samples pre-treated by nano-TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub>/UV ( $F_{1,4}=28.663$ ;  $p=0.006$ ), but at the end of the biological treatment the absorbance ratio at 465 nm increased for values significantly higher than the initial ones, for both fungi species (Table 3 and 4). Due to an increment in color in the samples treated with *P. chrysosporium*, significant differences between fungus species were recorded, but only in the sample pre-treated by nano-Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub>/UV (Table 5; Fig. 2).

As far as the absorbance ratio at 270 nm is considered no differences were recorded between fungi but a significant increment was observed in this parameter after 21 days of biological treatment for the OOMW samples pre-treated by nano-TiO<sub>2</sub>/UV and nano-Fe<sub>2</sub>O<sub>3</sub>/UV for *P. chrysosporium*, and for the samples pre-treated by nano-TiO<sub>2</sub>/UV and nano-Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub>/UV for *P. sajor caju* (Table 3 and 4; Fig. 2). The biological treatment did not contribute to any improvement in terms of aromatic compounds present in the OOMW. The observed increments in absorbance ratio at 465 and 275 nm for the treatments with *P. sajor caju* and *P. chrysosporium* species may have been related, at least in part, with the production of secondary metabolic compounds by the two species under study and its accumulation in the medium (Rocha-Santos et al. 2010). Previous studies reported the ability of *Trametes versicolor* to produce polysaccharides through submerge fermentation in different mediums (e.g. potato malt peptone medium, mushroom complete medium, yeast malt extract medium) (Cui and Chisti 2003; Kim et al. 2002). These polysaccharides are quite interesting, in addition to the fact that they act as an extracellular sheath surrounding the mycelium, it has been suggested that these extracellular polymers (e.g. glucan) can play a role in the lignin biodegradation process (Gutierrez et al. 1996; Krcmar et al. 1999). Although we did not record any improvement in terms of colour in our study, the biological treatment was in fact very efficient in terms of reduction of COD and TPC. A similar tendency was observed by other authors (Ferreira et al. 2008; Justino et al. 2009; Rocha-Santos et al. 2010). However, our results contrast with several other studies, reporting a high absorbance reduction at both wavelengths achieved by white-rot fungus for OOMW (Fountoulakis et al. 2002; Kissi et al. 2001; Ntougias et al. 2012). Yet, none of these studies made a chemical pre-treatment before the biological treatment with fungi. So we can assume that the chemical pre-treatment with NPs did



not favor a further reduction of absorbance ratio for both wavelengths by *P. chrysosporium* or *P. sajor caju*, probably due to the formation of intermediate colored compounds, such polymerized molecules (e.g. tanins). These compounds can absorb strongly to mycelia and to intracellular enzymes, causing inactivation, and jeopardizing their own degradation (Tsagaraki et al. 2007).

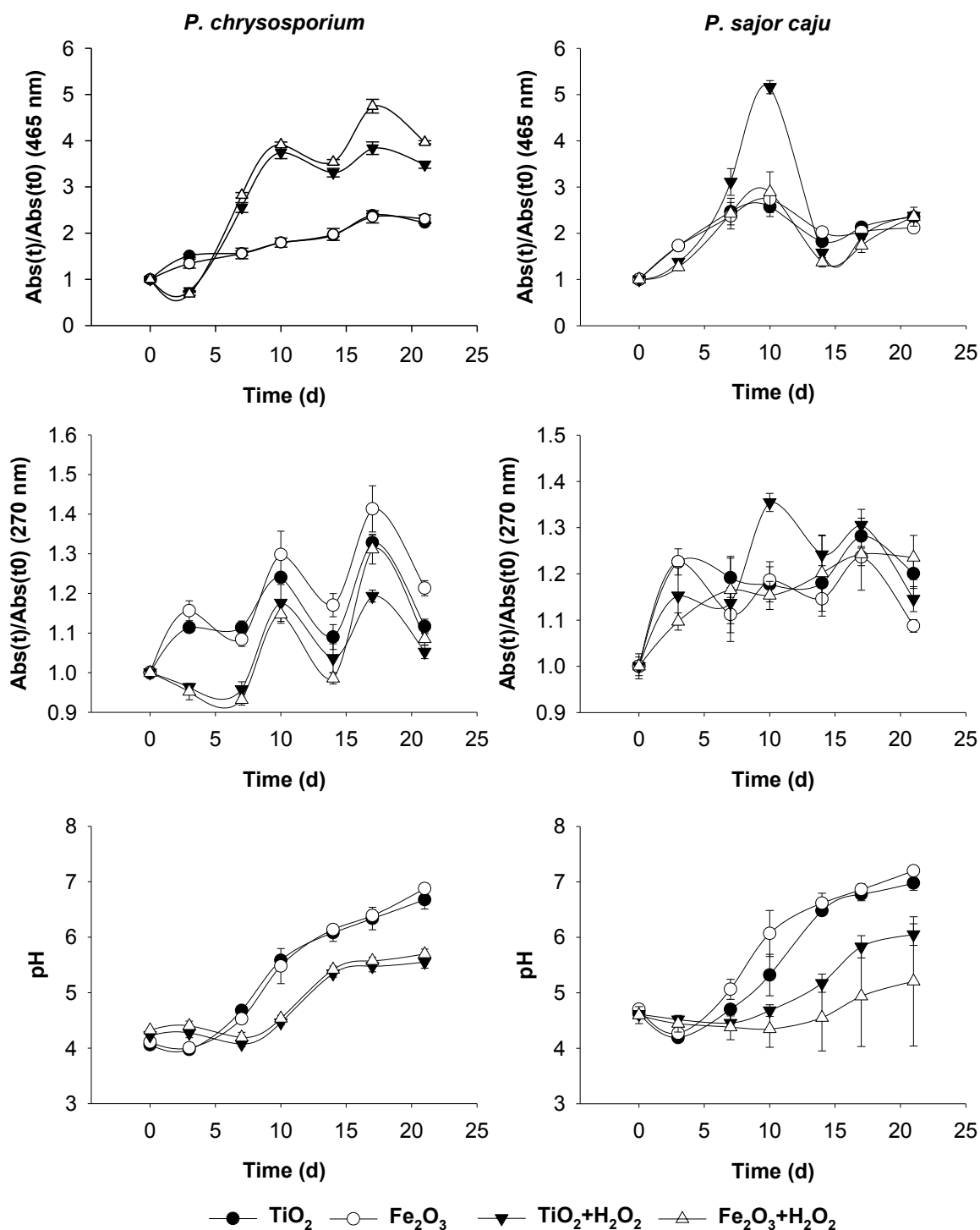


Figure IV.2- Absorbance ratio  $[(\text{Abs}(t)/\text{Abs}(t_0))]$  at 465 and 270 nm, and pH variation throughout time of OOMW pre-treated incubated with *P. chrysosporium* or *P. sajor caju* at 25°C and 120±10 rpm.

The pH variation throughout the biological treatment is represented in Fig. 2. The biological treatment increased the pH of the effluent samples with the different chemical pre-treatments reaching values between of 5.2 and 7.2 after 21 days of

treatment (Table 2). Significant differences between fungi in the samples pre-treated by nano-Fe<sub>2</sub>O<sub>3</sub>/UV and nano-TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub>/UV were recorded (Table 5), with higher values of pH (neutral pH values) in the samples treated with *P. sajor caju* (Fig. 2). The significant increment in pH, after the biological treatment with both fungi species, in almost all the pre-treated OOMW samples (Table 3 and 4), was probably due to the consumption of some organic acids, such as lactic and acetic acids, that are present in the OOMW (Fadil et al. 2003). Also the extracellular polysaccharides produced by fungi (previous discussed) can contribute to maintain pH to optimum values (4-5) for the activity of their ligninolytic enzymes (Krcmar et al. 1999; Raj et al. 2007). Hanafi et al. (2010) attributed the increase in pH to the reduction in phenolic content of effluent samples. Phenols are acidic in solution and when they are removed the acidity of the solution reduces (Hanafi et al. 2010). In fact, in our study, the samples pre-treated by nano-TiO<sub>2</sub>/UV and nano-Fe<sub>2</sub>O<sub>3</sub>/UV that presented the highest pH values (neutral pH) for both fungi also displayed the highest percentages of reduction in total phenolics content (Table 2).

Table 2 displays the fungus biomass in the beginning and at the end of the biological treatment and the respective growth rate for each species. Higher growth rates were achieved by *P. chrysosporium* with a significant increment of fungi biomass in all the OOMW pre-treated samples ( $F_{4,10}=24.952$ ;  $p<0.001$ ). However, for *P. sajor caju* exposed to the samples pre-treated with nano-TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub>/UV and nano-Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub>/UV no increment on fungi biomass was recorded (Table 2), in fact no significant differences were observed between the initial and the final biomass of *P. sajor caju* for all the OOMW pre-treated samples ( $F_{4,10}=3.441$ ;  $p=0.051$ ). *Pleurotus sajor caju* was the most sensitive species special in the samples pre-treated with both NPs and hydrogen peroxide. In the study of Massadeh and Modallal (2008) the growth of *P. sajor caju* was significantly lower when the OOMW was pretreated with hydrogen peroxide. The justification given by these authors suggest that hydrogen peroxide promoted a series of reactions changing the chemical composition of OOMW and reducing its nutrient value for the growth of *P. sajor caju* (Massadeh and Modallal 2008). The same justification can be applied in our case. We believe that we can put aside the assumption of being the NPs that are promoting a toxic effect since we filter the sample prior to biological treatment to remove the catalyst. Also, as we stated

before, the toxicity of these NPs was tested for a battery of species and they were selected precisely because they were less toxic (Chapter II). Nevertheless, despite the fact that no increment in biomass was recorded for *P. sajor caju* the percentages of reduction in terms of COD and TPC were equivalent for both fungi. However the ability of *P. sajor caju* to reduce the toxicity of the pre-treated samples was smaller than that of *P. chrysosporium* (Table 2). This indicates that the metabolic processes involved in toxicity removal were probably different between fungi. Probably *P. chrysosporium* used the components of OOMW as substrate and have adsorbed and metabolized these organic compounds within the cells. On the contrary *P. sajor caju* was probably sensitive to some components of OOMW so enhanced the production of extracellular enzymes to promote the degradation of these compounds in the medium. It is well known that white-rot fungi are responsible for the production of an array of extracellular enzymes with highly oxidative potential including manganese peroxidases (MnPs), lignin peroxidases (LiPs) and laccases which are capable of degrading a wide range of pollutants such as lignin. Alternatively to the extracellular enzymes, they have the potential to metabolize pollutants via intracellular enzymes, such as cytochrome P450 (Asgher et al. 2008; Harms et al. 2011; Subramanian and Yadav 2008). Indeed other authors have been reported that both processes can occur when using white-rot fungi (Yadav et al. 2001; Marco-Urrea et al. 2010; Yadav et al. 2001), however further studies are needed to understand the mechanisms involved in the degradation of OOMW.

In terms of COD, the 21 days of biological treatment promoted a significant decrease in this parameter, for all samples submitted to different pre-treatments and for both fungi (Table 3 and 4). The highest reductions in COD were recorded in the samples pre-treated with nano-TiO<sub>2</sub>/UV and nano-Fe<sub>2</sub>O<sub>3</sub>/UV, however, such reductions depended on the fungi used in the biological treatment and significant differences between fungi were noted for these two pre-treatments (Table 5). In the samples pre-treated by nano-TiO<sub>2</sub>/UV we reached reductions of COD of 43.6% with *P. chrysosporium* and 51.7% with *P. sajor caju*. While for the samples pre-treated by nano-Fe<sub>2</sub>O<sub>3</sub>/UV 51.6% and 60.4% reductions in this parameter were achieved, respectively (Table 2). As we can see from Table 2 the pre-treatment of the samples with nano-TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub>/UV and nano-Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub>/UV has decreased the ability of fungi

for reducing the COD content since the additional percentage of reduction promoted by the biological treatment was lower (10.4%-19.1%) (Table 2). The results may, in part, be explained by the remained organic intermediates produced during the pre-treatment with the systems using hydrogen peroxide. In the study of Justino et al. (2009) the treatability of OOMW by mean of photo-Fenton oxidation (with non-nano  $\text{Fe}^{2+}$ ) followed by biological treatment with *P. chrysosporium*, *T. versicolor* and *P. sajor caju* was assessed. In this study the biological treatment did not promote any reduction in terms of COD in an OOMW sample recently produced.

Table IV.2- Average  $\pm$  STDEV values of biomass, pH, COD, total phenolic content, % of reduction in COD and TPC and toxicity of OOMW samples pre-treated by chemical oxidation with nanoparticles, followed by biological treatment with *P. chrysosporium* or *P. sajor caju*. Toxicity data is expressed in % of dilution of the OOMW sample tested.

Chemical treatment	Fungi	Biomass (d.w g L <sup>-1</sup> )			pH		COD (g L <sup>-1</sup> )		Total phenolic content (TPC) (mg L <sup>-1</sup> )		Reduction (%)		EC50 <sub>30min</sub> (CI95)
		0 d	21 d	GR	0d	21d	0 d	21d	0d	21d	COD	TPC	
TiO <sub>2</sub> /UV	<i>P. chrysosporium</i>	0.90	1.97	2.2	4.3 $\pm$ 0.02	6.7 $\pm$ 0.2	15.8 $\pm$ 2.9	8.9 $\pm$ 0.6	132.5 $\pm$ 7.6	7.6 $\pm$ 0.1	43.6	94.7	21.78% (5.31-107.77)
Fe <sub>2</sub> O <sub>3</sub> /UV		0.90	2.09	2.3	4.4 $\pm$ 0.02	6.9 $\pm$ 0.1	18.1 $\pm$ 0.6	8.8 $\pm$ 0.4	120.7 $\pm$ 4.6	6.3 $\pm$ 0.7	51.6	95.5	36.97% (18.43-89.83)
TiO <sub>2</sub> /H <sub>2</sub> O <sub>2</sub> /UV		0.90	2.21	2.5	4.4 $\pm$ 0.01	5.5 $\pm$ 0.1	10.2 $\pm$ 0.3	8.3 $\pm$ 0.3	90.1 $\pm$ 9.0	28.3 $\pm$ 0.4	19.1	70.4	21.34% (6.74-78.93)
Fe <sub>2</sub> O <sub>3</sub> /H <sub>2</sub> O <sub>2</sub> /UV		0.90	2.21	2.5	4.5 $\pm$ 0.01	5.7 $\pm$ 0.1	10.4 $\pm$ 0.4	8.7 $\pm$ 0.1	105.0 $\pm$ 3.1	34.4 $\pm$ 1.8	16.7	66.6	31.85% (6.21-169.17)
		0 d	21 d	GR	0 d	21 d	0 d	21 d	0 d	21 d	COD	TPC	EC50 <sub>30min</sub>
TiO <sub>2</sub> /UV	<i>P. sajor caju</i>	1.11	1.53	1.4	4.3 $\pm$ 0.02	7.0 $\pm$ 0.1	15.8 $\pm$ 2.9	7.6 $\pm$ 0.1	132.5 $\pm$ 7.6	7.1 $\pm$ 0.5	51.7	95.0	4.59% (3.14-6.71)
Fe <sub>2</sub> O <sub>3</sub> /UV		1.11	1.49	1.3	4.4 $\pm$ 0.02	7.2 $\pm$ 0.1	18.1 $\pm$ 0.6	7.2 $\pm$ 0.4	120.7 $\pm$ 4.6	2.8 $\pm$ 0.7	60.4	98.3	8.70% (6.94-10.93)
TiO <sub>2</sub> /H <sub>2</sub> O <sub>2</sub> /UV		1.11	1.04	0.9	4.4 $\pm$ 0.01	6.1 $\pm$ 0.2	10.2 $\pm$ 0.3	8.9 $\pm$ 0.9	90.1 $\pm$ 9.0	11.9 $\pm$ 0.2	12.7	88.0	8.25% (7.50-9.13)
Fe <sub>2</sub> O <sub>3</sub> /H <sub>2</sub> O <sub>2</sub> /UV		1.11	0.78	0.7	4.5 $\pm$ 0.01	5.2 $\pm$ 1.2	10.4 $\pm$ 0.4	9.3 $\pm$ 0.2	105.0 $\pm$ 3.1	15.2 $\pm$ 6.8	10.4	85.7	3.03% (2.22-4.15)

Similar to COD, TPC decreased after the biological treatment. Significant percentages of reduction were recorded after 21 days of treatment, 94.7%, 95.5%, 70.4% and 66.6% after treatment with *P. chrysosporium* (Table 3), and 95.0%, 98.3%, 88.0% and 85.7 after treatment with *P. sajor caju* (Table 4), for nano-TiO<sub>2</sub>/UV, nano-Fe<sub>2</sub>O<sub>3</sub>/UV, nano-TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub>/UV and nano-Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub>/UV, respectively, being the lower reductions always recorded in the samples pre-treated by catalyst and oxidant together (Table 2). The use of H<sub>2</sub>O<sub>2</sub> in the pre-treatment contributes to increase the efficiency of the oxidation process giving smaller molecules, as we can see in Table 1,

however these compounds were probably more toxic inhibiting the efficiency of the fungi. On the contrary the pre-treatment using only the catalyst was not so efficient (Table 1) probably because the oxidation process was less aggressive; however it favors the action of fungi during the biological treatment. Further, the reduction in TPC of the pre-treated samples by nano-Fe<sub>2</sub>O<sub>3</sub>/UV, nano-TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub>/UV and nano-Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub>/UV was significantly higher for the biological treatment with *P. sajor caju* (Table 3). In the study performed by Justino et al. (2009) they recorded reductions in TPC of 61.8%, 60.7% and 48.3% for the treatments with *P. chrysosporium*, *T. versicolor* and *P. sajor caju*, respectively, after a pre-treatment of a recently produced OOMW with photo-Fenton oxidation. In summary, *P. sajor caju* was the less efficient species, in opposition to our observations in this study. It seems that the application of pre-treatments with NPs can increase the ability of fungi to reduce the TPC of OOMW, since we recorded higher reduction percentages; we also hypothesized that NPs played a role in controlling the native microbial flora of OOMW and somehow facilitated the action of fungi. The antibacterial activity of some NPs is well documented (Adams et al. 2006; Baek and An 2011).

The application of the biological treatment to the pre-treated samples improved some of the OOMW physical and chemical proprieties and also reduced samples toxicity (Table 2). The biological process with *P. chrysosporium* significantly reduced the toxicity of OOMW after the 21 days of treatment, independently of chemical pre-treatment applied to the sample (Table 3). However, for *P. sajor caju* only the samples pre-treated by nano-TiO<sub>2</sub>/UV, nano-Fe<sub>2</sub>O<sub>3</sub>/UV and nano-TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub>/UV displayed significant reductions for this parameter (Table 4). Nonetheless, significant differences between fungi were recorded for all the chemical pre-treatments, because higher reductions were always achieved after the treatment with *P. chrysosporium* (Table 5). The biological treatment with *P. sajor caju* was responsible for a slight reduction in toxicity namely: 2.6, 5.2, 3.1 and 1.4 times for the samples pre-treated by nano-TiO<sub>2</sub>/UV, nano-Fe<sub>2</sub>O<sub>3</sub>/UV, nano-TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub>/UV and nano-Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub>/UV, respectively (Table 2). On the other hand, the treatment with *P. chrysosporium* was able to promote remarkable reductions on toxicity, achieving increments in EC<sub>50</sub> values of 12.2 (nano-TiO<sub>2</sub>/UV), 22.1 (nano-Fe<sub>2</sub>O<sub>3</sub>/UV), 8.2 (nano-TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub>/UV) and 14.5 times (nano-Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub>/UV) (Table 2). Our data totally contrasts with those reported by Justino et

al. (2009). According to this study, the photo-Fenton oxidation followed by biological treatment with *P. chrysosporium* and *P. sajor caju* did not promote a reduction in the toxicity of the treated OOMW for *Daphnia longispina*, although reductions in terms of TPC were recorded. But similarly, in our study, *P. sajor caju* was not very efficient in terms of toxicity removal, and although best reductions in phenolic compounds were achieved, the growth rate of this species was very limited. As we stated before *P. sajor caju* and *P. chrysosporium* probably used different metabolic pathways during the degradation of OOMW. Different pathways lead to the formation of different metabolites (Yang et al. 2013) and this maybe responsible for the differences recorded in terms of toxicity in the end of the biological process. The end products of *P. sajor caju* did not contribute to the reduction of toxicity.

Table IV. 3- Results of two-way ANOVA testing the effects of biological treatment period (0 and 21 days) with *P. chrysosporium*, chemical pre-treatment (nano-TiO<sub>2</sub>/UV, nano-Fe<sub>2</sub>O<sub>3</sub>/UV, nano-TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub>/UV and nano-Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub>/UV) and their interaction on absorbance at 465 nm and 270 nm, pH, COD, total phenolic content and toxicity. One-way ANOVA followed by Tukey multiple comparison test ( $p < 0.05$ ) was used to evaluate statistical differences between 0 and 21 day for each pre-treatment applied.

<i>P. chrysosporium</i> Chemical pre-treatment versus time (0 and 21 days)					One Way ANOVA and Tukey multiple comparison tests
Endpoint	Source	df	F	P value	
Absorbance at 465 nm	Time	1,12	646.168	<0.001	TiO <sub>2</sub> /UV, Fe <sub>2</sub> O <sub>3</sub> /UV, TiO <sub>2</sub> /H <sub>2</sub> O <sub>2</sub> /UV, Fe <sub>2</sub> O <sub>3</sub> /H <sub>2</sub> O <sub>2</sub> /UV
	Chemical pre-treatment	5,12	43.786	<0.001	
	Interaction	5,12	7.621	0.002	
Absorbance at 270 nm	Time	1,12	35.374	<0.001	TiO <sub>2</sub> /UV, Fe <sub>2</sub> O <sub>3</sub> /UV
	Chemical pre-treatment	5,12	12.439	<0.001	
	Interaction	5,12	4.416	0.016	
pH	Time	1,12	1676.364	<0.001	TiO <sub>2</sub> /UV, Fe <sub>2</sub> O <sub>3</sub> /UV, TiO <sub>2</sub> /H <sub>2</sub> O <sub>2</sub> /UV, Fe <sub>2</sub> O <sub>3</sub> /H <sub>2</sub> O <sub>2</sub> /UV
	Chemical pre-treatment	5,12	31.261	<0.001	
	Interaction	5,12	57.919	<0.001	
COD	Time	1,12	144.725	<0.001	TiO <sub>2</sub> /UV, Fe <sub>2</sub> O <sub>3</sub> /UV, TiO <sub>2</sub> /H <sub>2</sub> O <sub>2</sub> /UV, Fe <sub>2</sub> O <sub>3</sub> /H <sub>2</sub> O <sub>2</sub> /UV
	Chemical pre-treatment	5,12	22.378	<0.001	
	Interaction	5,12	16.832	<0.001	
Phenolic compounds	Time	1,12	2767.349	< 0.001	TiO <sub>2</sub> /UV, Fe <sub>2</sub> O <sub>3</sub> /UV, TiO <sub>2</sub> /H <sub>2</sub> O <sub>2</sub> /UV, Fe <sub>2</sub> O <sub>3</sub> /H <sub>2</sub> O <sub>2</sub> /UV
	Chemical pre-treatment	5,12	102.372	< 0.001	
	Interaction	5,12	188.366	< 0.001	
Toxicity	Time	1,12	328.854	< 0.001	TiO <sub>2</sub> /UV, Fe <sub>2</sub> O <sub>3</sub> /UV, TiO <sub>2</sub> /H <sub>2</sub> O <sub>2</sub> /UV, Fe <sub>2</sub> O <sub>3</sub> /H <sub>2</sub> O <sub>2</sub> /UV
	Chemical pre-treatment	5,12	3.011	0.055	
	Interaction	5,12	4.455	0.016	



Table IV.4- Results of two-way ANOVA testing the effects of biological treatment period (0 and 21 days) with *P. sajor caju*, chemical pre-treatment (nano-TiO<sub>2</sub>/UV, nano-Fe<sub>2</sub>O<sub>3</sub>/UV, nano-TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub>/UV and nano-Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub>/UV) and their interaction on absorbance at 465 nm and 270 nm, pH, COD, total phenolic content and toxicity. One-way ANOVA followed by Tukey multiple comparison test (p < 0.05) was used to evaluate statistical differences between 0 and 21 day for each pre-treatment applied.

<i>P. sajor caju</i>					One Way ANOVA and Tukey multiple comparison tests
Chemical pre-treatment versus time (0 and 21 days)	Endpoint	Source	df	F	P value
Absorbance at 465 nm	Time		1,12	45.44 4	<0.001
	Chemical pre-treatment		5,12	7.735	0.002
	Interaction		5,12	1.203	0.365
Absorbance at 270 nm	Time		1,12	23.05 2	<0.001
	Chemical pre-treatment		5,12	2.114	0.134
	Interaction		5,12	0.905	0.509
pH	Time		1,12	74.55 9	<0.001
	Chemical pre-treatment		5,12	6.108	0.005
	Interaction		5,12	5.020	0.010
COD	Time		1,12	113.8 60	<0.001
	Chemical pre-treatment		5,12	4.815	0.012
	Interaction		5,12	28.89 9	<0.001
Phenolic compounds	Time		1,12	1562. 749	< 0.001
	Chemical pre-treatment		5,12	7.407	0.002
	Interaction		5,12	15.59 8	< 0.001
Toxicity	Time		1,12	111.0 58	< 0.001
	Chemical pre-treatment		5,12	10.12 4	<0.001
	Interaction		5,12	9.218	<0.001

Table IV. 5- Results of two-way ANOVA testing the effects of fungi (*P. chrysosporium* and *P. sajor caju*), chemical pre-treatment (nano-TiO<sub>2</sub>/UV, nano-Fe<sub>2</sub>O<sub>3</sub>/UV, nano-TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub>/UV and nano-Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub>/UV) and their interaction on absorbance at 465 nm and 270 nm, pH, COD, total phenolic content and toxicity. One-way ANOVA followed by Tukey multiple comparison test ( $p < 0.05$ ) was used to evaluate statistical differences between fungi to each pre-treatment applied.

Fungi versus chemical pre-treatment					One Way ANOVA and Tukey multiple comparison tests
Endpoint	Source	df	F	P value	
Absorbance at 465 nm	Fungi	1,16	9.055	0.008	Fe <sub>2</sub> O <sub>3</sub> /H <sub>2</sub> O <sub>2</sub> /UV
	Chemical pre-treatment	3,16	3.284	0.048	
	Interaction	3,16	4.181	0.023	
Absorbance at 270 nm	Fungi	1,16	1.699	0.211	-----
	Chemical pre-treatment	3,16	0.510	0.681	
	Interaction	3,16	2.316	0.114	
pH	Fungi	1,16	31.969	<0.001	Fe <sub>2</sub> O <sub>3</sub> /UV, TiO <sub>2</sub> /H <sub>2</sub> O <sub>2</sub> /UV
	Chemical pre-treatment	3,16	132.304	<0.001	
	Interaction	3,16	1.686	0.213	
COD	Fungi	1,16	3.415	0.083	TiO <sub>2</sub> /UV, Fe <sub>2</sub> O <sub>3</sub> /UV
	Chemical pre-treatment	3,16	4.514	0.018	
	Interaction	3,16	8.023	0.002	
Phenolic compounds	Fungi	1,16	66.715	< 0.001	Fe <sub>2</sub> O <sub>3</sub> /UV, TiO <sub>2</sub> /H <sub>2</sub> O <sub>2</sub> /UV, Fe <sub>2</sub> O <sub>3</sub> /H <sub>2</sub> O <sub>2</sub> /UV
	Chemical pre-treatment	3,16	93.387	< 0.001	
	Interaction	3,16	5.968	< 0.001	
Toxicity	Fungi	1,16	156.429	< 0.001	TiO <sub>2</sub> /UV, Fe <sub>2</sub> O <sub>3</sub> /UV, TiO <sub>2</sub> /H <sub>2</sub> O <sub>2</sub> /UV, Fe <sub>2</sub> O <sub>3</sub> /H <sub>2</sub> O <sub>2</sub> /UV
	Chemical pre-treatment	3,16	4.865	0.014	
	Interaction	3,16	4.331	0.020	

#### 4.4 Conclusions

In spite of all efforts OOMW is still of great concern mainly in the Mediterranean countries and several efforts have been made to develop successfully treatments. Our results shown that combined chemical oxidation with NPs and biological treatment with fungi is appropriate for removing COD, TPC and toxicity. Both fungi promoted remarkable reductions in the TPC but not so great in COD reduction after the chemical pre-treatment. *P. chrysosporium* was the most efficient in reducing the OOMW toxicity. However, the biological treatment was not able to promote absorbance reductions at 465 and 270 nm. For color reduction the chemical treatment alone is an interesting solution with reductions of almost 50%, nevertheless the final effluent still highly toxic to *V. fischeri*. The most promising methodology is the chemical pre-treatment with nano-Fe<sub>2</sub>O<sub>3</sub>/UV followed by the biological treatment with *P. chrysosporium*, taking into accounts both physic and chemical parameters as well the integrity of the receiving freshwater ecosystem, as toxicity was significantly reduced. The use of NPs in the pre-treatment of OMW apparently were responsible for less strong oxidative processes or plays an important role in controlling the native microbial flora of OOMW favoring the action of fungi in the biological treatment.

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# Chapter V

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Treatment of mining and kraft pulp mill effluents with nano-TiO<sub>2</sub> and nano-Fe<sub>2</sub>O<sub>3</sub> photocatalysis



## Treatment of mining and kraft pulp mill effluents with nano-TiO<sub>2</sub> and nano-Fe<sub>2</sub>O<sub>3</sub> photocatalysis.

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### Abstract

Due to the lack of adequate treatment solutions for all the types of wastewaters and of proper disposal systems, huge amounts of industrial wastewaters are being discharged into aquatic environments. A vast number of pollutants, such as organic and inorganic chemicals, have been described as causing significant effects on the environment. In this scenario, nanotechnology is a promising technology due to the unique proprieties of the new materials produced, such as size, surface area, adsorptivity and photocatalytic potential, which can be helpful in facing the challenges of wastewater treatment. In this paper, we focus our study on the treatment of two real hazardous wastewaters, kraft pulp mill effluent and mining effluent, by photocatalysis using two nanomaterials (TiO<sub>2</sub> and Fe<sub>2</sub>O<sub>3</sub>). The results showed that for the organic effluent the systems TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub>/UV and Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub>/UV at pH 3 and, 2h of UV radiation, had the best performances with removal percentages of 93% for colour, 68% for aromatic compounds and 89% for COD. For the inorganic effluent results indicated that the treatments were only able to significantly remove three metals, the Zn, Al and Cd, being the highest reductions attained with 1.0 g L<sup>-1</sup> of TiO<sub>2</sub>/UV and Fe<sub>2</sub>O<sub>3</sub>/UV, respectively. Also in terms of reducing the toxicity to the bacterium *Vibrio fischeri* the most promising treatments were those using the oxidant and catalyst together for the organic effluent, and 1.0 g L<sup>-1</sup> of TiO<sub>2</sub> and Fe<sub>2</sub>O<sub>3</sub> for the inorganic effluent. In fact, the increase in catalyst concentrations increases the toxicity reduction, especially by nano-TiO<sub>2</sub>. According to our study the use of nanomaterials (NMs) in wastewater treatment seems to have a better result in organic effluents than in effluents with high content of metals and metalloids. In this paper two NMs were for the first time tested and compared in the photocatalytic treatment of a mining effluent.

### 5.1 Introduction

One of the main priorities worldwide is the protection and conservation of natural water resources since is being recognized that water scarcity is increasing.

Water resources are facing profound changes due to the spread of a wide range of contaminants in surface water and groundwater (WHO, 2013). Moreover, large amounts of wastewater from agriculture, domestic and industrial sources are being generated and discharged into the aquatic environment. Several pollutants, such as organic compounds like dyes, phenols, chlorinated and aromatic compounds, pesticides, volatile organic compounds, as well as, inorganic compounds like metals, persist in wastewaters and attain aquatic resources, causing serious environmental problems (Velhal et al., 2012; Vinu and Madras, 2012). Consequently, it is imperative to find proper treatments capable of resolving, or at least reduce, to acceptable levels the pollutants of these effluents, which tend to increase in parallel with economic growth.

The high variety of contaminants in wastewater makes their treatment a difficult and expensive task. Moreover, some of this contaminants, such as organic compounds and metal ions, have recalcitrant properties or are not bio-transformed, persisting in the environment (Poyatos et al., 2010). Advanced oxidation processes (AOPs) such as semiconductor photocatalysis are considered a strong technology for wastewater treatment with several applications to environmental pollutants degradation. This type of technology has the advantage of completely destroy a variety of hazardous compounds without secondary waste generation (Kabra et al., 2004). These treatments are based on the formation of reactive oxygen species, like hydroxyl radicals, which have the potential of oxidizing organic compounds, sometimes leading to its complete mineralization, or of reducing toxic metals converting them in their less-toxic or nontoxic metallic states (Chen and Ray, 2001; Kabra et al., 2004; Machulek Jr. et al., 2013). Since in photocatalysis the reactions occur on the surface of the semiconductor, the surface properties of the catalyst such as surface area and particle size play an important role. Thus the application of nanomaterials as catalysts can greatly increase the efficiency of this treatment.

Nanomaterials (NMs) are defined as unbound particles or aggregates/agglomerates particles, where at least 50% of them have one nanoscale dimension, with size ranging from 1 to 100 nm (European Community, 2011). The use of NMs as semiconductors in photocatalysis has several advantages. Decreasing particles size the specific surface area increases, increasing the adsorption ability. In

addition, the surface is highly reactive (due to their large radii of curvature) with high activity for photocatalysis (Mohmood et al., 2013; Theron et al., 2008). Furthermore, many studies have already proved the efficiency of NMs for the degradation of a variety of organic pollutants such as phenols, chlorinated aromatic compounds and dyes, as well the reduction of several metals like Cu(II), Hg(II), Cd(II), Zn(II) and Ni(II) (Prasse and Ternes, 2010). For example, Dutta et al. (2005) showed the complete removal of arsenic from drinking water to values below the ones recommended by the World Health Organization ( $10 \mu\text{g L}^{-1}$ ) through oxidation of As(II) to As(V) followed by adsorption using two available TiO<sub>2</sub> suspensions [Degussa P25 ( $\approx 30 \text{ nm}$ ) and Hombikat UV100 ( $< 10 \text{ nm}$ )]. In another study, Zelmanov and Semiat (2008), investigated the treatment of two organic compounds, ethylene glycol and phenol, by photocatalysis with iron-based nanocatalysts. The Fenton-like reaction using iron (3) oxide-based nanocatalysts in the presence of hydrogen peroxide was able to efficiently remove both organic contaminants without the need of UV light (Zelmanov and Semiat, 2008).

In this paper, we focus our study on the treatment of two real hazardous wastewaters, kraft pulp mill effluent and mining effluent, by photocatalysis using two NMs (herein mentioned as nano-TiO<sub>2</sub> and nano-Fe<sub>2</sub>O<sub>3</sub>). The photodegradation process was carried out using treatments such as TiO<sub>2</sub> and Fe<sub>2</sub>O<sub>3</sub>-assisted photocatalysis (nano-TiO<sub>2</sub>/UV and nano-Fe<sub>2</sub>O<sub>3</sub>/UV), nano-TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub>/UV and nano-Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub>/UV. For the organic effluent the effect of pH (3, 7 and 11), catalyst dose (0.25, 0.50, 0.75 and  $1.0 \text{ g L}^{-1}$ ) and the addition of an oxidant concentration (H<sub>2</sub>O<sub>2</sub>: 10, 25, 50 and  $75 \text{ mM}$ ) to the photocatalyst system, was investigated. The efficiency of the treatment was assessed through colour (465 nm), aromatic compounds (270nm) and chemical oxygen demand (COD) reductions. In the inorganic effluent the effects of operational parameters such as the presence of H<sub>2</sub>O<sub>2</sub> and catalyst dose (0.25, 0.50, 0.75 and  $1.0 \text{ g L}^{-1}$ ) in the reduction of metal contents were assessed. The pH effect was not assessed for the inorganic effluent since the raise of pH can directly influence the precipitation of several metals. Inductively coupled plasma-mass spectrometry (ICP-MS) was used to determine the concentration of seven elements (sulphur, copper, zinc, aluminium, arsenic, lead, cadmium) before and after the treatment. For both effluents the bioluminescence inhibition test with *Vibrio fischeri* was also performed to assess the reduction of toxicity after the treatment processes. To the best of our knowledge, this

is the first study comparing the efficiency of two NMs in the photocatalytic treatment of an organic and an inorganic effluent.

## 5.2 Materials and Methods

### 5.2.1 Organic and inorganic effluent

The organic effluent was collected from an *Eucalyptus globulus* bleached kraft pulp mill, after secondary treatment (herein mentioned as raw effluent). The raw effluent was collected in dark glass bottles and kept at 4°C until analysis. The effluent sample was characterized for pH, chemical oxygen demand (COD) and absorbance values at 465 nm for colour and 270 nm for aromatic compounds (Table 1).

Table V.1- Average values ( $\pm$  standard deviation) of pH, COD and absorbance, at 270 and 465 nm, for the raw effluent of the bleached kraft pulp mill.

	pH	COD (mg L <sup>-1</sup> )	Abs 465 nm	Abs 270 nm
Raw effluent	8.83 $\pm$ 0.05	391 $\pm$ 2	0.260 $\pm$ 0.001	0.367 $\pm$ 0.005

The inorganic effluent was collected from the São Domingos (Portugal) mine area, an abandoned cupric pyrite mine located in the Iberian Pyrite Belt. The sulphide oxidation process produces acid mine leachates (or acid mine drainage – AMD) rich in sulphates and metals (Table 2), which are responsible for serious environmental problems affecting the biological communities (Álvarez-Valero et al., 2007; Lopes et al., 1999). The water sample used in the present study was collected from a settlement pond. The water was collected into plastic containers (1.5 L) previously rinsed with 10 % HCl. The samples were transported under refrigeration to the laboratory and stored at -20°C until use.

Table V.2- Metals and metalloids concentration (average  $\pm$  standard deviation) and pH in the original effluent from São Domingos mine.

pH	Metals and metalloids						
	Sulfur (S) (mg L <sup>-1</sup> )	Copper (Cu) (mg L <sup>-1</sup> )	Zinc (Zn) (mg L <sup>-1</sup> )	Aluminum (Al) (mg L <sup>-1</sup> )	Arsenic (As)( $\mu$ g L <sup>-1</sup> )	Lead (Pb) ( $\mu$ g L <sup>-1</sup> )	Cadmium (Cd) ( $\mu$ g L <sup>-1</sup> )
2.58 $\pm$ 0.07	402.30 $\pm$ 1.8	1.02 $\pm$ 0.05	48.00 $\pm$ 1.6	74.00 $\pm$ 0.7	1.18 $\pm$ 0.15	13.50 $\pm$ 1.05	56.17 $\pm$ 1.47

### 5.2.2 Nano-catalysts and hydrogen peroxide

The two NPs were purchased as nanopowders; titanium (IV) oxide – TiO<sub>2</sub> (anatase, particle size < 25nm, 99.7% metal basis) from Sigma Aldrich and iron (III) oxide – Fe<sub>2</sub>O<sub>3</sub> (nanorods, particle information: d=40-130nm, l=250-600nm, average particle size 85x425 nm, 99% purity) from Nanostructured & Amorphous Materials Inc. (Houston, Texas, USA). Hydrogen peroxide (30%, analytical grade) was purchased from Fisher Scientific.

### 5.2.3 Photocatalytic experiments

#### Organic effluent

The photocatalysis process was conducted with nano-TiO<sub>2</sub>/UV, nano-Fe<sub>2</sub>O<sub>3</sub>/UV, nano-TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub>/UV and nano-Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub>/UV systems. Photodegradation treatments were carried out, at 20°C, using 50 ml of kraft pulp mill effluent in 100 ml glass beakers. Three replicates were prepared per treatment. The suspensions with catalyst were placed on an orbital shaker (100 rpm) overnight. After this period, the UV lamp (Spectroline XX15F/B, Spectronics Corporation, NY, USA, peak emission at 312 nm) was switch on, and left for 2 hours. During the irradiation period, agitation was maintained by magnetic stirring to keep the suspension homogenous. Different sets of experiments were performed. Firstly, the influence of pH was assessed using 1.0 g L<sup>-1</sup> of nano-TiO<sub>2</sub> or nano-Fe<sub>2</sub>O<sub>3</sub> (TiO<sub>2</sub>/UV and Fe<sub>2</sub>O<sub>3</sub>/UV) at pH 3.0, 7.0 and 10.0 adjusted with HCl (0.6 N) and NaOH (0.6 N). Secondly, the influence of different concentrations of nano-TiO<sub>2</sub> and nano-Fe<sub>2</sub>O<sub>3</sub> was studied (0.25, 0.50, 0.75 and 1.0 g L<sup>-1</sup>) at fixed pH of 3.0. Finally, the influence of different concentrations of H<sub>2</sub>O<sub>2</sub> was also investigated (10, 25, 50 and 75 mM), since, according to the literature, the presence of strong oxidant species (e.g. H<sub>2</sub>O<sub>2</sub> and O<sub>3</sub>) can greatly enhance the photodegradation process (Chiou et al., 2008; Garcia et al., 2007; Ghaly et al., 2001; Jamil et al., 2011). In the nano-TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub>/UV and nano-Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub>/UV systems, the concentration of the NPs was fixed in 0.75 g L<sup>-1</sup> of nano-TiO<sub>2</sub> or nano-Fe<sub>2</sub>O<sub>3</sub> at pH 3.0, and different volumes of H<sub>2</sub>O<sub>2</sub> were tested. After the UV exposure the samples were centrifuged (14000 rpm for 20 min) and filtered through GF/F (Ø47mm, Whatman) glass-fiber filter to remove the catalyst particles before analysis. In the treatments with H<sub>2</sub>O<sub>2</sub>, samples were withdrawn for analysis of chemical oxygen demand (COD) and to perform ecotoxicity

tests. This was done after the addition of sodium hydroxide (0.6 N) and after heating in a bath (100°C), for 4h, to completely eliminate residual hydrogen peroxide. It has been shown that the rate of H<sub>2</sub>O<sub>2</sub> decomposition (to oxygen and water) increases several folds as pH and temperature increase (Qiang et al., 2002).

### **Inorganic effluent**

The experiments were conducted using 50 ml of mining effluent in 100 ml glass beakers at 20°C. Three replicates were established per treatment. The suspensions with catalyst were placed on an orbital shaker (100 rpm) overnight. After this period, the UV lamp was switch on, and left for 2 hours. During the irradiation period, agitation was maintained by magnetic stirring to keep the suspension homogenous. The influence of different concentrations of nano-TiO<sub>2</sub> and nano-Fe<sub>2</sub>O<sub>3</sub> (nano-TiO<sub>2</sub>/UV and nano-Fe<sub>2</sub>O<sub>3</sub>/UV) was studied (0.25, 0.50, 0.75 and 1.0 g L<sup>-1</sup>). The influence of the presence of an oxidant (H<sub>2</sub>O<sub>2</sub>) was also investigated keeping the concentration of catalyst fixed (1.0 g L<sup>-1</sup>). After the UV exposure the samples were centrifuged (14000 rpm for 20 min) and filtered through GF/F (Ø47mm, Whatman) glass-fiber filter to remove the catalyst particles before analysis. In the treatments with H<sub>2</sub>O<sub>2</sub>, samples were withdrawn for ecotoxicity tests after the addition of sodium hydroxide (0.6 N) and after heating in a bath (100°C), for 4h, to completely eliminate residual hydrogen peroxide.

#### **5.2.4 Post-treatment evaluations**

The absorbance measurements before and after the treatments were performed using a UV/VIS spectrophotometer (Shimadzu 1800) at wavelengths associated with aromatic (270 nm) and coloured (465 nm) compounds. The pH was measured with a pH meter (pH meter 330 from WTW). Total phenolic content was determined by the colorimetric method of Folin–Ciocalteau (Mulinacci et al., 2001). Inductively coupled plasma-mass spectrometry (ICP-MS) was used to determine the concentration of seven elements (S, Cu, Zn, Al, As, Pb, Cd) before and after the treatment. The short-term toxicity of all the samples, before and after treatment for both effluents, was assessed following the 81.9% Basic Test protocol provided by the supplier, using a Microtox Model 500 Analyser (Azur Environmental, 1998). The endpoint measured in



the Microtox assay was the decrease in the intensity of light emitted by the luminescent marine bacteria – *Vibrio fischeri* – after 5, 15 and 30 min of exposure to several dilutions of the effluent (using a dilution factor of 2x), after previous osmotic and pH (6-8) adjustment of the samples.

#### 5.2.5 Statistical analysis

The EC<sub>50</sub> values and their corresponding 95% confidence intervals for *V. fischeri*, were calculated using the MicrotoxOmni® software version V1.18 for the three exposure periods (Azur Environmental, 1998). Whenever, it was not possible to obtain and EC<sub>50</sub>, the highest effect (percentage of bioluminescence inhibition) recorded is reported. To test for statistically significant differences among different treatments and control (raw effluent) for each parameter analysed (colour, aromatic compounds, COD, and metals and metalloids concentration) one-way analysis of variance (ANOVA) were employed, followed by Dunnett's test ( $\alpha=0.05$ ). To find out if there were any significant differences in terms of colour, aromatic compounds, COD and elements concentration removal due to the concentration of nano-TiO<sub>2</sub> or nano-Fe<sub>2</sub>O<sub>3</sub>, or even between the presence or absence of H<sub>2</sub>O<sub>2</sub>, a One Way ANOVA was conducted followed by the Tukey multiple comparison test for each parameter. This analysis was performed using the software SigmaPlot version 11.0 (Systat Software, Inc., 2008).

### 5.3. Results and Discussion

#### 5.3.1 Pulp mill effluent

##### 5.3.1.1 Effects of initial pH

The effect of pH on photocatalysis efficiency was investigated given that it can influence the charge on the catalyst surface, size of catalyst aggregates as well the positions of conductance and valence bands (Chong et al., 2010). Acidic (pH 3), neutral (pH 7) and alkaline (pH 11) pH were tested to find out the most suitable pH for applying nano-TiO<sub>2</sub>/UV and nano-Fe<sub>2</sub>O<sub>3</sub>/UV treatments and to obtain good results in terms of reduction of colour, aromatic compounds and COD of the pulp mill effluent. The concentration of the catalyst was maintained constant (1.0 g L<sup>-1</sup>) in all the experiments. Figure 1 shows the percentages of reduction, for the three parameters

assessed, at different pH levels after the treatment with nano-TiO<sub>2</sub>/UV (a) and nano-Fe<sub>2</sub>O<sub>3</sub>/UV (b). Higher percentages of reduction for both treatments were achieved at pH 3. For the nano-TiO<sub>2</sub>/UV (a) system at pH 3 we reached significant percentages of reduction of 87.22%, 59.67% and 62.57% for colour ( $F_{3,8}=507.446$ ,  $p<0.001$ ), aromatic compounds ( $F_{3,8}=738.927$ ,  $p<0.001$ ) and COD ( $F_{3,8}=5.754$ ,  $p=0.021$ ), respectively. We also recorded significant differences from the control for colour ( $F_{3,8}=507.446$ ,  $p<0.001$ ) and aromatic compounds ( $F_{3,8}=738.927$ ,  $p<0.001$ ) at pH 7 and pH 11, with reductions of 32.38% and 22.07% at pH 7 and 17.62% and 8.81% at pH 11. In the treatments involving nano-Fe<sub>2</sub>O<sub>3</sub>/UV the percentages of reduction did not go beyond 35.48% ( $F_{3,8}=12.934$ ,  $p=0.002$ ), 22.52% ( $F_{3,8}=14.289$ ,  $p=0.001$ ) and 31.31% ( $F_{3,8}=1.520$ ,  $p=0.282$ ) for colour, aromatic compounds and COD, respectively, and the maximum was recorded at acidic pH. In a similar study, Rodrigues et al. (2008) reported that the optimum pH value for the photocatalytic oxidation (nano-TiO<sub>2</sub> (P25)/UV) of paper and pulp mill wastewater was 3.0. Also Li et al. (2011) using nano-Fe<sub>2</sub>O<sub>3</sub> ( $\approx 20$  nm) showed that the maximum degradation of a straw pulp and paper mill effluent was obtained at pH 3. Similar results were also observed in the treatment of olive mill wastewater (Kallel et al., 2009; Ugurlu and Karaoglu, 2010). These results can be explained by the point of zero charge ( $pH_{pzc}$ ) of the catalyst, i.e. the pH at which the surface charge becomes neutral, which occurs in the range of pH 6.3 – 7.8 for both nano-TiO<sub>2</sub> and nano-Fe<sub>2</sub>O<sub>3</sub> (French et al., 2009; Hu et al., 2006; Park et al., 2008). At pH values above the  $pH_{pzc}$  the catalyst surface is negatively charged. Below the  $pH_{pzc}$  the surface is positively charge, and anion adsorption occurs by electrostatic attraction (Ahmed et al., 2010; Umar and Aziz, 2013). The adsorption of water or hydroxide ions promote the generation of hydroxyl radicals which attack organic pollutants (with negative charge) in the pulp mill effluent that are also adsorbed on the surface of the catalyst (Chong et al., 2010; Rodrigues et al., 2008).

Since it was confirmed that pH 3 showed the best results, recording the higher reduction percentages for both catalysts, further experiments were carried out at pH 3.

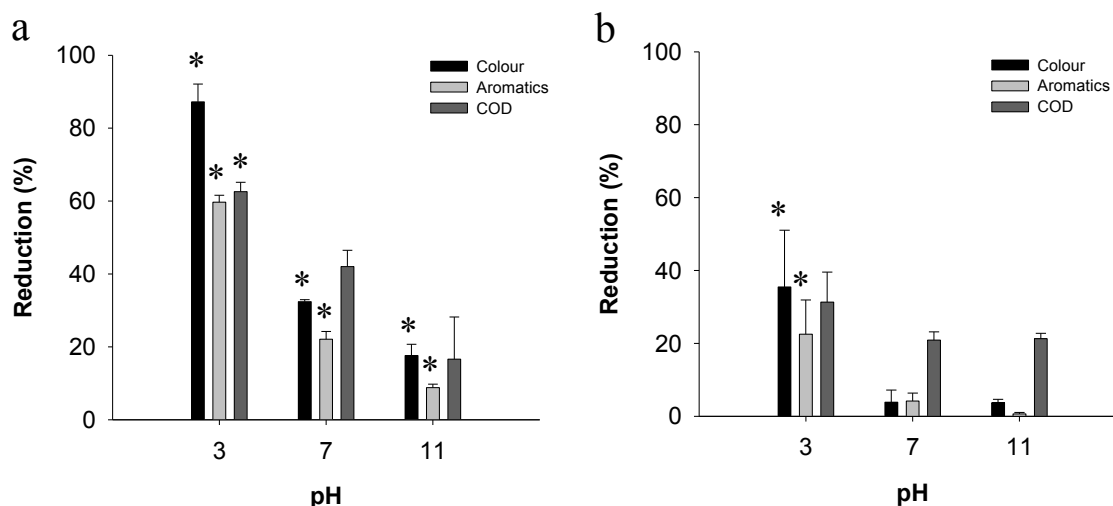


Figure V.1- Effect of pH (pH 3, 7 and 11) on colour, aromatic compounds and COD reduction after treatment of pulp mill effluent with nano-TiO<sub>2</sub>/UV (a) and nano-Fe<sub>2</sub>O<sub>3</sub> (b) at 1.0 g L<sup>-1</sup> of catalyst (nano-TiO<sub>2</sub> or nano-Fe<sub>2</sub>O<sub>3</sub>) and 2 hours of exposure to UV light. Asterisk stand for statistical significant differences from the control, for corresponding treatments (Dunnett tests; p<0.05).

### 5.3.1.2 Effects of catalyst (nano-TiO<sub>2</sub> and nano-Fe<sub>2</sub>O<sub>3</sub>) dose

One of the main factors affecting degradation studies is the amount of catalyst used. In order to avoid the use of an excessive concentration of catalyst several doses (2-fold factor between each dose) of nano-TiO<sub>2</sub> and nano-Fe<sub>2</sub>O<sub>3</sub> (0.25 to 1.0 g L<sup>-1</sup>) were added to pulp mill effluent at pH 3. Figure 2 shows the results of different loadings of nano-TiO<sub>2</sub> (a) and nano-Fe<sub>2</sub>O<sub>3</sub> (b) on the reduction (%) of colour, aromatic compounds and COD after 2 hours of UV irradiation at pH 3. The results clearly show that for the treatment with nano-TiO<sub>2</sub>/UV the increase of the catalyst dose from 0.25 to 1.0 g L<sup>-1</sup> increased the percentage of colour, aromatic compounds and COD removal achieving reductions of 87.22% ( $F_{4,10}=420.944$ ,  $p<0.001$ ), 59.67% ( $F_{4,10}=847.567$ ,  $p<0.001$ ) and 62.57% ( $F_{4,10}=17.290$ ,  $p<0.001$ ), respectively, at the highest concentration of the catalyst (Fig. 2a). The increasing efficiency recorded was caused by the increase in the availability of active sites on catalyst surface, with the increase in its concentration, thus raising the number of adsorption sites and the number of •OH radicals (Ahmed et al., 2010; Ibhadon and Fitzpatrick, 2013; Umar and Aziz, 2013).

Figure 2b revealed that when we use nano-Fe<sub>2</sub>O<sub>3</sub> as catalyst the reduction of aromatic compounds increased significantly by increasing catalyst loading achieving

22.52% of reduction with  $1.0 \text{ g L}^{-1}$  ( $F_{4,10}=9.677$ ,  $p=0.002$ ). However, for colour and COD, the beneficial effect of increasing catalyst concentration fades out at  $0.75 \text{ g L}^{-1}$  and  $0.50 \text{ g L}^{-1}$ , respectively, since a further increase does not contribute to higher reductions. At  $0.75 \text{ g L}^{-1}$  of nano- $\text{Fe}_2\text{O}_3$  a significant reduction of 43.30% for colour ( $F_{4,10}=15.906$ ,  $p<0.001$ ) was achieved while at  $0.50 \text{ g L}^{-1}$  a significant reduction of 43.99% for COD ( $F_{4,10}=5.473$ ,  $p=0.013$ ) was recorded. Other studies using photocatalytic degradation to treat organic effluents also exhibit the same dependency on catalyst dose (Catalkaya and Kargi, 2008; Chiou et al., 2008; Ghaly et al., 2011; Li et al., 2011). The excess of catalyst concentration can promote aggregation reducing the active surface area available and decrease the light penetration due to the shielding effect (Ahmed et al., 2010; Jagadale et al., 2012; Umar and Aziz, 2013), thus decreasing efficiency. Although, for  $0.75 \text{ g L}^{-1}$  of nano- $\text{TiO}_2$  the efficacy was slightly lower than that for  $1.0 \text{ g L}^{-1}$  (Fig. 2a), Tukey's test proved that the percent removals of colour ( $p=0.152$ ) and COD ( $p=0.097$ ) for these two concentrations are similar. So in this study further experiments were performed using  $0.75 \text{ g L}^{-1}$  of catalyst (nano- $\text{TiO}_2$  or nano- $\text{Fe}_2\text{O}_3$ ), at pH 3.

As can be seen in Fig. 1 and 2 (a and b) nano- $\text{TiO}_2$  seemed to be more effective than nano- $\text{Fe}_2\text{O}_3$  in the reduction of all the parameters assessed. The observed results can be explained by two phenomena. The first one is the differences in particle size of both catalysts. Being the size of nano- $\text{TiO}_2$  ( $< 25 \text{ nm}$ ) NPs smaller than the size of nano- $\text{Fe}_2\text{O}_3$  ( $\approx 85 \times 425 \text{ nm}$ ) NPs they will have a higher surface to volume ratio, and therefore more active sites will be available. Since all the reactions occur in the surface of the particles the efficiency to degrade organic compounds was higher for nano- $\text{TiO}_2$ . The second phenomenon is related to the band gap. The band gap for nano- $\text{TiO}_2$  anatase and nano- $\text{Fe}_2\text{O}_3$  is 3.2 eV (Kumar and Devi, 2011) and 2.2 eV (Chirita and Grozescu, 2009), respectively. When the band gap is smaller the distance between the conduction band and the valence band is smaller therefore the recombination electron-hole is higher decreasing the efficiency of the catalyst. Hence, the photocatalytic activity of nano- $\text{TiO}_2$  is higher than the nano- $\text{Fe}_2\text{O}_3$  (Botía et al., 2012; Singaravelan and Palanisamy, 2010).

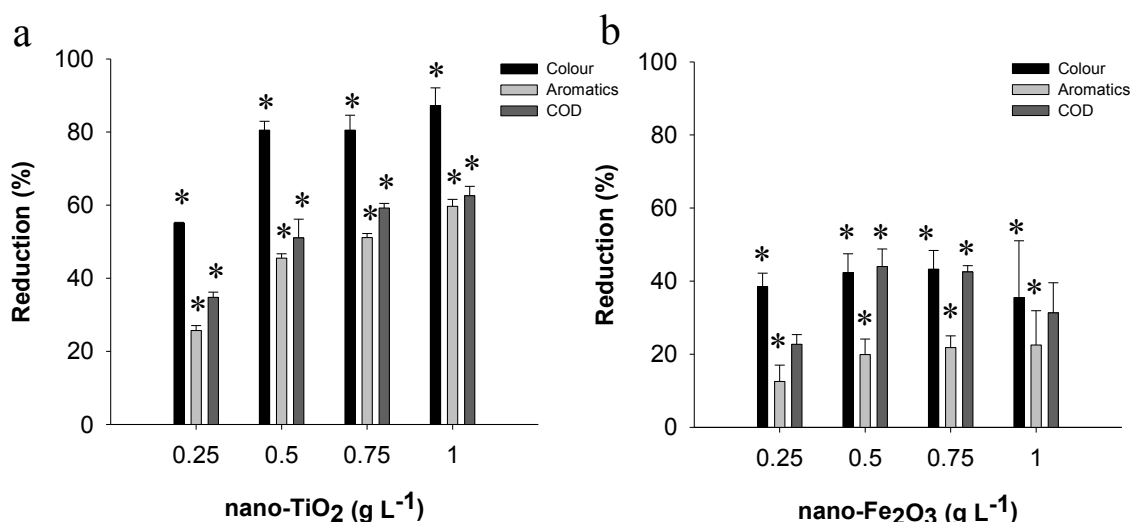


Figure V.2- Effect of nano-TiO<sub>2</sub> (a) and nano-Fe<sub>2</sub>O<sub>3</sub> (b) dose on colour, aromatic compounds and COD reduction after treatment of pulp mill effluent at pH 3 and 2 hours of exposure to UV light. Asterisk stand for statistical significant differences from the control, for corresponding treatments (Dunnett tests;  $p < 0.05$ ).

### 5.3.1.3 Effect of addition of H<sub>2</sub>O<sub>2</sub>

In the photocatalytic process, oxidizing agents, such as hydrogen peroxide or ozone, have a great deal of influence in the degradation of organic compounds. To elucidate the effect of adding H<sub>2</sub>O<sub>2</sub> on the photocatalytic degradation of pulp mill wastewater some experiments were carried out by varying the initial H<sub>2</sub>O<sub>2</sub> concentration, while maintaining the pH (3), and the dose of the catalyst (0.75 g L<sup>-1</sup>) and 2h of UV. As shown in Fig. 3, the addition of H<sub>2</sub>O<sub>2</sub> had a beneficial effect on the removal of colour, aromatic compounds and COD of the pulp mill wastewater, when compared with the treatments using only catalyst (nano-TiO<sub>2</sub>/UV and nano-Fe<sub>2</sub>O<sub>3</sub>/UV) (Fig 2). This beneficial effect was more pronounced specially between the treatments nano-Fe<sub>2</sub>O<sub>3</sub>/UV and nano-Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub>/UV, as removal percentages increased significantly from 43.30%, 21.80% and 42.54% to a maximum of 92.93% (with 10 mM of H<sub>2</sub>O<sub>2</sub>), 68.39% (with 10 mM of H<sub>2</sub>O<sub>2</sub>) and 81.67% (with 75 mM of H<sub>2</sub>O<sub>2</sub>) for colour (Tukey's test:  $p < 0.001$ ), aromatic compounds (Tukey's test:  $p < 0.001$ ) and COD (Tukey's test:  $p < 0.001$ ), respectively. These can be explained by the effect of the additional production of reactive radical intermediates ( $\bullet\text{OH}$ ) produced by H<sub>2</sub>O<sub>2</sub>; also these radicals can act as electron scavengers inhibiting the electron-hole recombination of the catalyst (Jagadale et al., 2012; Shanthi and Kuzhalosai, 2012). The addition of H<sub>2</sub>O<sub>2</sub>

to the system nano-TiO<sub>2</sub>/UV was not so beneficial, only a significant increment in the removal percentage of colour (Tukey's test:  $p=0.009$ ), from 80.52% to 93.30% (with 75 mM of H<sub>2</sub>O<sub>2</sub>), and COD (Tukey's test:  $p<0.001$ ), from 62.57% to 89.77% (with 25 mM of H<sub>2</sub>O<sub>2</sub>), was recorded.

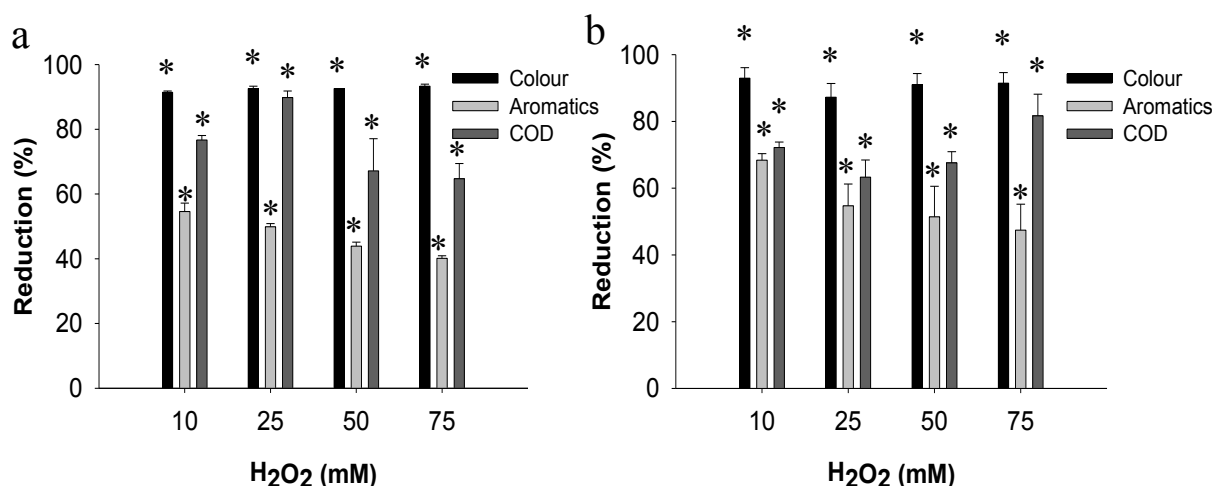


Figure V.3- Effect of H<sub>2</sub>O<sub>2</sub> loading in the process nano-TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub>/UV (a) and nano-Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub>/UV (b) on colour, aromatic compounds and COD reduction at pH 3, 0.75 g L<sup>-1</sup> catalyst loading and 2 hours of exposure to UV light. Asterisk stand for statistical significant differences from the control, for corresponding treatments (Dunnett tests;  $p<0.05$ ).

All the concentrations of H<sub>2</sub>O<sub>2</sub> promoted significant reductions (%) for both systems, nano-TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub>/UV (colour:  $F_{4,10}=21833.050$ ,  $p<0.001$ ; aromatics:  $F_{4,10}=592.805$ ,  $p<0.001$ ; COD:  $F_{4,10}=51.588$ ,  $p<0.001$ ) and nano-Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub>/UV (colour:  $F_{4,10}=35.696$ ,  $p<0.001$ ; aromatics:  $F_{4,10}=53.241$ ,  $p<0.001$ ; COD:  $F_{4,10}=19.038$ ,  $p<0.001$ ), compared to the control (raw effluent) for all the parameters tested. The results showed (Fig 3a) that increasing H<sub>2</sub>O<sub>2</sub> concentration from 10 to 75 mM a positive effect on the percentage of colour removal (91.44% to 93.30%) was observed for the system nano-TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub>/UV. This was expected since the number of hydroxyl radicals produced is directly proportional to the hydrogen peroxide concentration (Li et al., 2011; Shanthi and Kuzhalosai, 2012). However, for aromatic compounds the increase of H<sub>2</sub>O<sub>2</sub> concentration was not followed by an increase in the % of reduction of these compounds. In opposition the hydrogen peroxide decreased the efficiency of both systems to degrade aromatic compounds. The same tendency was reported by Li et al. (2011) when they treat pulp and paper mill wastewater with nano-Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub>/UV.

These authors find that the rate of COD removal increased with addition of H<sub>2</sub>O<sub>2</sub> from 1‰ to 5‰ (v/v), however after 5‰ the removal rate began to decrease (Li et al., 2011). The excess of H<sub>2</sub>O<sub>2</sub> can itself react with •OH radicals formed ( $\text{OH}\bullet + \text{H}_2\text{O}_2 \rightarrow \text{HO}_2\bullet + \text{H}_2\text{O}_2$ ) or be adsorbed at the surface of catalyst competing with the organic compounds and consequently reduce the efficiency of the treatment. Also it might be due to self-break down of H<sub>2</sub>O<sub>2</sub> into oxygen and water ( $2\text{H}_2\text{O}_2 \rightarrow 2\text{H}_2\text{O} + \text{O}_2$ ) and to the recombination of hydroxyl radicals ( $2\text{OH}\bullet \rightarrow \text{H}_2\text{O}_2$ ) (Li et al., 2011; Shanthi and Kuzhalosai, 2012).

In this study the Fenton-like process (nano-Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub>/UV) and the photocatalytic process nano-TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub>/UV revealed to be the best treatments for pulp mill effluent. At the best condition of pH, catalyst and oxidant we recorded removal percentages of 92.93% for colour, 68.39% for aromatic compounds and 81.67% for COD with nano-Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub>/UV, and 93.30%, 54.59% and 89.77%, respectively, with nano-TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub>/UV. Similar results were obtained by Li et al. (2011) after the treatment of pulp and paper mill wastewater by nano-Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub>/UV (size of α-Fe<sub>2</sub>O<sub>3</sub> ≈ 20 nm). After 3h of UV, at pH 3 with 1.0 g L<sup>-1</sup> of catalyst and 5% (v/v) of H<sub>2</sub>O<sub>2</sub>, these authors observed a reduction percentage of 93.1% for COD, however no ecotoxicological evaluation of the final effluent was done. Although the removal of COD was slight higher in this study compared to our results it can probably be explained by the amount of catalyst and oxidant used. The amount of catalyst and oxidant used by Li et al. (2011) was higher than in our study (0.75 g L<sup>-1</sup> of catalyst and 25 mM of H<sub>2</sub>O<sub>2</sub>), also more time of exposure to UV was needed. However, the increase of energy consumption and reagents may not offset the increased efficiency. Another study, performed by Ghaly et al. (2011), tested the treatability of paper mill wastewater through solar photocatalytic oxidation using a synthesized nano-TiO<sub>2</sub> (unknown size). Using a nano-TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub>/UV system with 0.75 g L<sup>-1</sup> of catalyst, 2 ml L<sup>-1</sup> of oxidant and pH 6.5, 77.9% COD removal was achieved after 180 min of solar irradiation (Ghaly et al., 2011). The reduction percentage was lower compared to our study, despite the use of a high oxidant concentration and the prolonged exposure to solar irradiation.

#### 5.3.1.4 Toxicity to *V. fischeri*

The toxicity of the raw effluent and treated kraft pulp mill effluent was evaluated using a bioassay with *V. fischeri* (Table 1). For almost all treated samples it was not possible to determine an EC<sub>50</sub> value, since bioluminescence inhibition was lower than 50%, or slightly above 50% only in the highest concentration of the treated effluent tested. In these cases only the highest effect (HE) observed is reported (Table 1). In the treatments using only the catalyst (nano-TiO<sub>2</sub>/UV and nano-Fe<sub>2</sub>O<sub>3</sub>/UV), a slight reduction occurred with 0.50 and 0.75 g L<sup>-1</sup> of nano-TiO<sub>2</sub>, as well with 0.25 g L<sup>-1</sup> and 1.00 g L<sup>-1</sup> of nano-Fe<sub>2</sub>O<sub>3</sub>. However, for the other concentrations tested (0.25 and 1.00 g L<sup>-1</sup> of nano-TiO<sub>2</sub>, and 0.50 and 0.75 g L<sup>-1</sup> of nano-Fe<sub>2</sub>O<sub>3</sub>) the treated effluent became more toxic than the raw effluent (Table 1). According to some authors, although the great efficiency of the photocatalytic process in providing high reductions in COD and colour (also proven by our results), the possible formation of secondary metabolites (organic compounds that are partially oxidized or non-oxidized) during the treatment turns the effluent more toxic than the initial one (Oller et al., 2011; Pereira et al., 2009; Rizzo, 2011; Tambosi, 2006). Pereira et al. (2009) reported an exhaustive evaluation of the toxicity of a secondary bleached kraft pulp mill effluent, after tertiary treatment with photo-Fenton oxidation, using a battery of freshwater species. Extremely low EC<sub>50</sub> values for *V. fischeri*, *Raphidocelis subcapitata* and *Daphnia magna* were recorded after tertiary treatment with photo-Fenton oxidation. These results were mainly attributed to the presence of unknown secondary metabolites resulting from the oxidation of organic compounds, and also due to the presence of inhibitory compounds (e.g. metals and sulphur compounds) that persist in the effluent even after the photo-Fenton treatment (Pereira et al., 2009). In terms of efficiency, the systems associating nano-TiO<sub>2</sub> or nano-Fe<sub>2</sub>O<sub>3</sub> with H<sub>2</sub>O<sub>2</sub> were the best, with the highest removal rates for the physicochemical parameters evaluated. Also in terms of toxicity nano-TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub>/UV and nano-Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub>/UV promoted the highest reductions (Table 1). Actually, for nano-Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub>/UV system the increase of H<sub>2</sub>O<sub>2</sub> concentration reduces the toxicity of kraft pulp mill effluent to *V. fischeri* gradually (Table 1). The best reductions were achieved for the samples treated with nano-Fe<sub>2</sub>O<sub>3</sub> and 75 mM of H<sub>2</sub>O<sub>2</sub> and also with 50 mM, as well with nano-TiO<sub>2</sub> and 25 mM of H<sub>2</sub>O<sub>2</sub>, since we recorded the lowest HE. This can be explained by the strong oxidation reactions promoted by these treatments that



were responsible for the almost complete mineralization of the toxic organic compounds. Undoubtedly nano-Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub>(75mM)/UV was the best treatment removing almost all the toxicity of the effluent (HE<sub>30min</sub>=6.50%; Table 1).

Table V.3- Values of EC<sub>50</sub> recorded for bioluminescence inhibition expressed in effluent percentage, and corresponding 95% confidence intervals (CI95), or the highest effect expressed in bioluminescence inhibition percentage for OOMW samples after the treatments at 5, 15, and 30 min exposure of *V. fischeri* (HE – highest effect).

	EC <sub>50</sub> (%) / Highest effect		
	5 min	15 min	30 min
<b>Raw effluent</b>	<b>HE - 41.17%</b>	<b>74.85% (15.43-255.4)</b>	<b>64.32% (8.94-487.7)</b>
<b>UV/TiO<sub>2</sub> 0.25 g L<sup>-1</sup></b>	64.06% (9.83-434.7)	39.07% (7.59-217.4)	32.17% (8.88-116.8)
<b>UV/TiO<sub>2</sub> 0.50 g L<sup>-1</sup></b>	<b>HE - 32.04%</b>	<b>HE - 36.37%</b>	<b>HE - 39.39%</b>
<b>UV/TiO<sub>2</sub> 0.75 g L<sup>-1</sup></b>	<b>HE - 31.55%</b>	<b>HE - 34.36%</b>	<b>HE - 39.90%</b>
<b>UV/TiO<sub>2</sub> 1.00 g L<sup>-1</sup></b>	52.48% (7.88-349.5)	57.23% (12.17-269.0)	53.85% (10.21-284.1)
<b>UV/Fe<sub>2</sub>O<sub>3</sub> 0.25 g L<sup>-1</sup></b>	<b>HE - 41.64%</b>	<b>HE - 49.82%</b>	67.07% (26.59-169.2)
<b>UV/Fe<sub>2</sub>O<sub>3</sub> 0.50 g L<sup>-1</sup></b>	76.94% (11.67-564.2)	44.45% (10.92-202.3)	35.08% (11.35-119.6)
<b>UV/Fe<sub>2</sub>O<sub>3</sub> 0.75 g L<sup>-1</sup></b>	63.79% (15.07-268.8)	38.65% (11.57-135.2)	35.08% (11.94-106.8)
<b>UV/Fe<sub>2</sub>O<sub>3</sub> 1.00 g L<sup>-1</sup></b>	<b>HE - 37.30%</b>	<b>HE - 36.02%</b>	<b>HE - 40.96%</b>
<b>UV/TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub> 10 mM</b>	<b>HE - 39.06%</b>	<b>HE - 50.64%</b>	49.79% (20.06-123.5)
<b>UV/TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub> 25 mM</b>	<b>HE - 24.12%</b>	<b>HE - 24.78%</b>	<b>HE - 20.84%</b>
<b>UV/TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub> 50 mM</b>	<b>HE - 28.96%</b>	<b>HE - 20.61%</b>	<b>HE - 21.48%</b>
<b>UV/TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub> 75 mM</b>	54.33% (17.96-155.1)	42.56% (11.90-156.4)	53.42% (35.86-105.0)
<b>UV/Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub> 10 mM</b>	<b>HE - 43.41%</b>	<b>HE - 48.95%</b>	<b>HE - 50.51%</b>
<b>UV/Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub> 25 mM</b>	<b>HE - 20.84%</b>	<b>HE - 25.61%</b>	<b>HE - 31.33%</b>
<b>UV/Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub> 50 mM</b>	<b>HE - 19.86%</b>	<b>HE - 19.88%</b>	<b>HE - 22.91%</b>
<b>UV/Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub> 75 mM</b>	<b>HE - 13.96%</b>	<b>HE - 12.76%</b>	<b>HE - 6.50%</b>

### 5.3.2 Mining effluent

#### 5.3.2.1 Effects of catalyst dose

From the seven elements (S, Cu, Zn, Al, As, Pb, Cd) analysed we only recorded significant reductions between the control and the treated samples in terms of zinc, aluminium and cadmium concentrations. The removal of Zn, Al and Cd was investigated by varying the initial concentration of nano-TiO<sub>2</sub> or nano-Fe<sub>2</sub>O<sub>3</sub>. Figure 4 shows the effect of nano-TiO<sub>2</sub> and nano-Fe<sub>2</sub>O<sub>3</sub> dosage on the removal of Zn, Al and Cd. With the increase of the catalyst (nano-TiO<sub>2</sub> or nano-Fe<sub>2</sub>O<sub>3</sub>) dose from 0.50 to 1.00 g L<sup>-1</sup>, the removal efficiency of Zn increased significantly ( $F_{10,55}=10.513$ ,  $p<0.001$ ) (Fig. 4) reaching reductions of 14.9% and 12.5% with 1.0 g L<sup>-1</sup> of nano-TiO<sub>2</sub> (Dunnett's test:  $p<0.05$ ) and nano-Fe<sub>2</sub>O<sub>3</sub> (Dunnett's test:  $p<0.05$ ), respectively. The increase of sites

available for adsorption in the surface of the catalyst, as the concentration increases, promotes the increase of removal efficiency (Al-Saad et al., 2012). Nevertheless, we recorded significant reductions in Zn for all the treatments tested (Fig. 4). However for Al we only recorded a significant reduction of 3.5% with  $1.00 \text{ g L}^{-1}$  of nano- $\text{Fe}_2\text{O}_3$  ( $F_{10,55}=2.481$ ,  $p=0.016$ ) (Fig. 4b). Thus, the increase in catalyst concentration did not favour the removal process. Also, for Cd the increment on catalyst dose did not promote any improvement on the removal efficiency. Significant reductions ( $F_{10,55}=2.444$ ,  $p=0.017$ ) for Cd were recorded after the treatment with  $0.25 \text{ g L}^{-1}$  of nano- $\text{TiO}_2$  (6.8%; Dunnett's test:  $p<0.05$ ),  $0.25 \text{ g L}^{-1}$  of nano- $\text{Fe}_2\text{O}_3$  (6.2%; Dunnett's test:  $p<0.05$ ) and  $0.50 \text{ g L}^{-1}$  of nano- $\text{Fe}_2\text{O}_3$  (6.5%; Dunnett's test:  $p<0.05$ ).

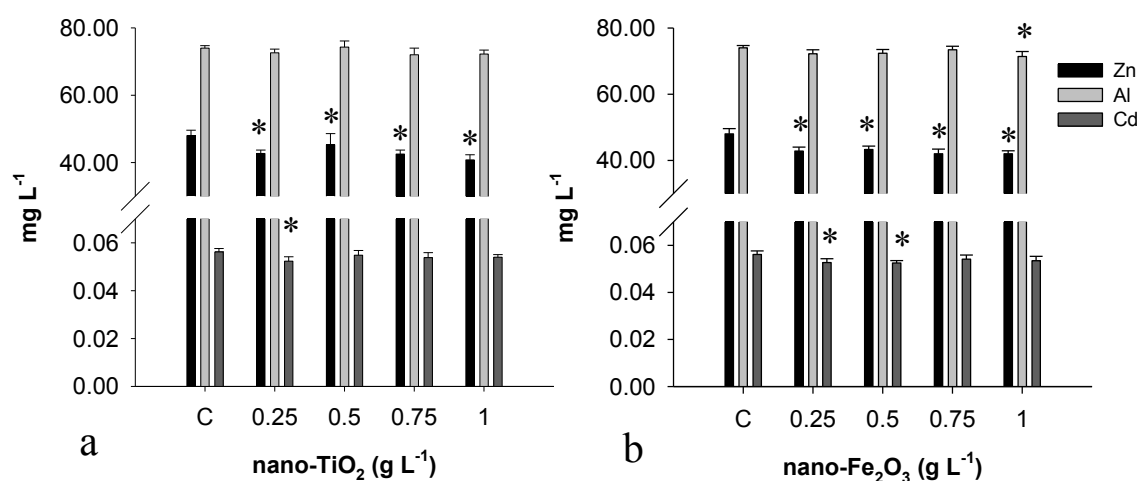


Figure V.4. Effect of nano- $\text{TiO}_2$  (a) and nano- $\text{Fe}_2\text{O}_3$  (b) dose on the concentration of zinc, aluminum and cadmium after treatment of a mining effluent after 2 hours of exposure to UV light. Error bars are the standard deviations. Asterisks indicate significant differences from the control ( $p<0.05$ ).

The removal percentages for all the elements tested in our study were not very high. As mentioned before for the organic wastewater pH played an important role on photocatalysis. For example, in the study of Samarghandi et al. (2007) the treatment of synthetic samples of Cd with nano- $\text{TiO}_2$  (30 nm)/UV at acidic pH (pH 3.5) the percentage of reduction for Cd did not go behind 21%, however raising the pH until 11 the removal percentage increase to 94.1%. However, the increase in the removal efficiency was probably not due to the presence of the catalyst, but by the simple fact that most of the metal ions can readily precipitate by raising the pH of a solution

(Wang et al., 2004). The same dependency was also observed in the study of Mahdavi et al. (2013). They investigated the potential of TiO<sub>2</sub> (12 nm), MgO (24 nm) and Al<sub>2</sub>O<sub>3</sub> (11 nm) nanoparticles as sorbents for the removal of Cd<sup>2+</sup>, Cu<sup>2+</sup>, Ni<sup>2+</sup>, and Pb<sup>2+</sup> from aqueous solutions. The maximum removal of the four ions was found to be pH 5 and pH 4 for nano-Al<sub>2</sub>O<sub>3</sub> (31%, 8.5%, 11.7%, and 18.7% for Cd<sup>2+</sup>, Cu<sup>2+</sup>, Ni<sup>2+</sup>, and Pb<sup>2+</sup>, respectively) and nano-TiO<sub>2</sub> (37.5%, 14.9%, 47.8%, and 14.9% for Cd<sup>2+</sup>, Cu<sup>2+</sup>, Ni<sup>2+</sup>, and Pb<sup>2+</sup>, respectively), respectively. While for nano-MgO the pH had no obvious effect. The results observed by these authors with nano-TiO<sub>2</sub> and nano-Al<sub>2</sub>O<sub>3</sub> were attributed to the competitive adsorption between H<sup>+</sup> ions and the metals on solution in acidic pH (Mahdavi et al., 2013). However, in the study of Thakur et al. (2014), the efficiency of the process depends not only on the pH but also on the metal under study. They found that the photoreduction of Cr<sup>6+</sup> and Cu<sup>2+</sup> was favourable in acidic conditions, while for Zn<sup>2+</sup> and Ni<sup>2+</sup> at alkaline pH (Thakur et al., 2014). The pH has a fundamental role in the photocatalytic reduction of metals; on one hand it influences the surface charge of the catalyst and its adsorption ability, but on the other hand it affects the solubility of the metals ions, the degree of ionization and the quantity of ions with opposite charge on the surface of the adsorbent (Atieh, 2011). The inherent pH of 2.6 of the effluent may be the reason for the low removal percentages recorded in our study. Another reason may be the competitive adsorption to the surface of catalyst between all the metals present in the effluent. Siboni et al. (2012) studied the simultaneous removal of Cr(VI) and Ni(II) from a water by nano-TiO<sub>2</sub>(21 nm)/UV using 1.0 g L<sup>-1</sup> of catalyst at pH 7. The efficiency of the process decrease during the 120 min of UV when both metals are present (Siboni et al., 2012).

#### **5.3.2.2 Effect of addition of H<sub>2</sub>O<sub>2</sub>**

The effect of the addition of H<sub>2</sub>O<sub>2</sub> to the nano-TiO<sub>2</sub>/UV and nano-Fe<sub>2</sub>O<sub>3</sub>/UV systems is represented in Fig. 5. Significant reductions were recorded for Zn ( $F_{10,55}=10.513$ ,  $p<0.001$ ) with both nano-TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub>/UV (Dunnett's test:  $p<0.05$ ) and nano-Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub>/UV (Dunnett's test:  $p<0.05$ ). We also recorded significant reduction for Cd ( $F_{10,55}=2.444$ ,  $p=0.017$ ) after the treatment with nano-Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub>/UV (Dunnett's test:  $p<0.05$ ). However, only for Zn and Cd the addition of H<sub>2</sub>O<sub>2</sub> to the nano-Fe<sub>2</sub>O<sub>3</sub>/UV

system had a beneficial effect. A slight increase in the removal percentage of Zn from 12.5% using nano-Fe<sub>2</sub>O<sub>3</sub> (1.0 g L<sup>-1</sup>)/UV to 13.5% with nano-Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub>/UV was recorded. An increase in the removal efficiency of Cd was also achieved. Without the addition of H<sub>2</sub>O<sub>2</sub> to the system nano-Fe<sub>2</sub>O<sub>3</sub> (1 g L<sup>-1</sup>)/UV we did not record significant differences from the control for Cd, however when H<sub>2</sub>O<sub>2</sub> was added to this system the reduction increased to 5.1% and it was significantly different from the control. Samarghandi et al. (2007) also showed that the removal efficiency of metals could be improved by the addition of H<sub>2</sub>O<sub>2</sub> to the TiO<sub>2</sub>/UV system. At pH 3.5 the removal percentage of Cd increase from 21% (using 0.80 g L<sup>-1</sup> of TiO<sub>2</sub> and UV) to 26% by adding 3 ml L<sup>-1</sup> of H<sub>2</sub>O<sub>2</sub> (Samarghandi et al., 2007).

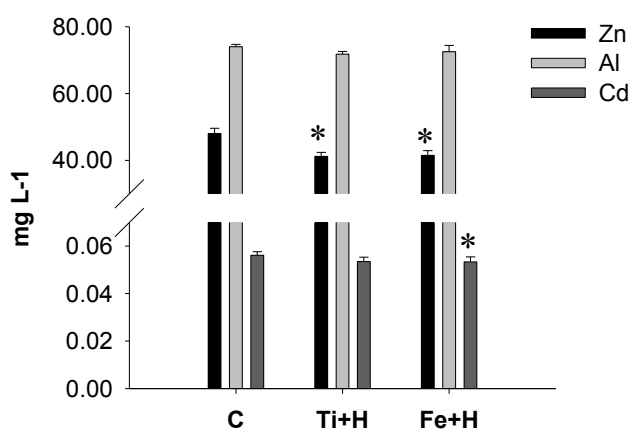


Figure V.5- Effect of the addition of H<sub>2</sub>O<sub>2</sub> to nano-TiO<sub>2</sub> (Ti+H) or nano-Fe<sub>2</sub>O<sub>3</sub> (Fe+H) system on the concentration of zinc, aluminium and cadmium after treatment of a mine effluent after 2 hours of exposure to UV light. Error bars are the standard deviations. Asterisks indicate significant differences from the control ( $p < 0.05$ ).

### 5.3.2.3 Toxicity to *V. fischeri*

Although only three out of seven elements were significantly removed from the mining effluent by photocatalytic reduction, a decrease in the toxicity of the acid mine drainage to *V. fischeri* was observed after the treatment (Table 2). For the treatments using only nano-TiO<sub>2</sub> or nano-Fe<sub>2</sub>O<sub>3</sub> toxicity reductions were recorded for all the tested concentrations of the catalysts. However, the best reductions were achieved by using 1.0 g L<sup>-1</sup> of nano-TiO<sub>2</sub> or of nano-Fe<sub>2</sub>O<sub>3</sub> (Table 2). The ICP-MS analysis only gives us the

total concentration of a specific element; however we can have different oxidation states for each one. The oxidation state can determine the behaviour (e.g. solubility and bioavailability) and toxicity of metals in the environment (Reeder et al., 2006). The redox reactions produced during photocatalysis can control the oxidation state of metals and then controlling the mobility and the toxicity of these elements. Several studies have reported, for example, the photocatalytic reduction of Cr(VI), which is mobile and highly toxic, into Cr(III), which is immobile and less harmful (Barrera-Díaz et al., 2012). The reductions in terms of toxicity recorded in our study for the treatment with nano-TiO<sub>2</sub>/UV and nano-Fe<sub>2</sub>O<sub>3</sub>/UV at different concentrations of catalyst where probably due to the photocatalytic reduction of the elements present in the wastewater to a less toxic state. In fact, as we increase the concentration of nano-TiO<sub>2</sub> the toxicity to *V. fischeri* reduces progressively (Table 2). The same tendency is also observed for nano-Fe<sub>2</sub>O<sub>3</sub> however only at the concentration of 0.50 g L<sup>-1</sup> (Table 2). Also as stated before the oxidation state of a metal influence their bioavailability, the photocatalytic process probably reduced the metals to a state where the metal cannot be adsorbed by *V. fischeri*.

Table V.4- Values of EC<sub>50</sub> for bioluminescence inhibition expressed in effluent percentage, and corresponding 95% confidence intervals (CI95), or the highest effect expressed in bioluminescence inhibition percentage for mining effluent samples after the treatments at 5, 15, and 30 min exposure of *V. fischeri* (HE – highest effect).

	EC <sub>50</sub> (%) / Highest effect		
	5 min	15 min	30 min
<b>Raw effluent</b>	<b>58.06% (37.76-89.30)</b>	<b>14.32% (12.12-17.02)</b>	<b>3.68% (3.02-4.49)</b>
<b>UV/TiO<sub>2</sub> 0.25 g L<sup>-1</sup></b>	<b>HE - 31.01%</b>	<b>HE - 34.57%</b>	<b>HE - 40.98%</b>
<b>UV/TiO<sub>2</sub> 0.50 g L<sup>-1</sup></b>	<b>HE - 38.10%</b>	<b>HE - 40.72%</b>	38.26% (24.59-62.47)
<b>UV/TiO<sub>2</sub> 0.75 g L<sup>-1</sup></b>	<b>HE - 26.68%</b>	<b>HE - 27.98%</b>	<b>HE - 28.00%</b>
<b>UV/TiO<sub>2</sub> 1.00 g L<sup>-1</sup></b>	<b>HE - 15.99%</b>	<b>HE - 16.41%</b>	<b>HE - 27.38%</b>
<b>UV/Fe<sub>2</sub>O<sub>3</sub> 0.25 g L<sup>-1</sup></b>	<b>HE - 34.07%</b>	<b>HE - 34.27%</b>	<b>HE - 36.28%</b>
<b>UV/Fe<sub>2</sub>O<sub>3</sub> 0.50 g L<sup>-1</sup></b>	<b>HE - 33.84%</b>	<b>HE - 37.92%</b>	<b>HE - 44.06%</b>
<b>UV/Fe<sub>2</sub>O<sub>3</sub> 0.75 g L<sup>-1</sup></b>	<b>HE - 43.90%</b>	24.20% (14.44-40.61)	14.23% (8.33-24.35)
<b>UV/Fe<sub>2</sub>O<sub>3</sub> 1.00 g L<sup>-1</sup></b>	<b>HE - 17.43%</b>	<b>HE - 21.00%</b>	<b>HE - 29.29%</b>
<b>UV/TiO<sub>2</sub>/H<sub>2</sub>O<sub>2</sub></b>	47.80% (13.28-182.5)	31.86% (12.94-93.51)	15.30% (7.65-36.32)
<b>UV/Fe<sub>2</sub>O<sub>3</sub>/H<sub>2</sub>O<sub>2</sub></b>	31.73% (9.63-281.6)	19.86% (7.54-77.35)	6.70% (4.96-9.29)

## 5.4 Conclusion

Despite all the efforts that have been made in the last decades to develop effective treatment processes several organic and inorganic pollutants still persist in industrial wastewaters, reaching the aquatic environment. Advanced oxidation processes based in photocatalysis were applied to treat the organic content of a kraft pulp mill effluent as well the inorganic content of a mining wastewater. The study reveals that the efficiency of photocatalysis with NMs for the treatment of industrial wastewater depended upon the nature of the chemicals found in the effluent, since better results were recorded for the organic effluent than for the mine effluents with a high content of metals for the physic-chemical parameters. For the organic effluent, both catalysts (nano-TiO<sub>2</sub> and nano-Fe<sub>2</sub>O<sub>3</sub>), under optimal conditions, can effectively reduce the colour, aromatic compounds, COD and the toxicity. However, nano-TiO<sub>2</sub> alone was a more efficient catalyst than nano-Fe<sub>2</sub>O<sub>3</sub>, but the addition of a proper amount of hydrogen peroxide to the system with nano-Fe<sub>2</sub>O<sub>3</sub> can improve the reductions percentages for all the parameters tested for both catalysts. Regarding the inorganic effluent, although we only recorded significant reductions in three of the seven metals present in the wastewater, remarkable reductions in terms of toxicity were achieved. This probably means that the photocatalytic reduction of metals occurred, making them less bioavailable, thus decreasing their toxicity.

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# Chapter VI

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**Toxicity of solid residues resulting from wastewater treatment with  
nanomaterials**



## Toxicity of solid residues resulting from wastewater treatment with nanomaterials

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### Abstract

Nanomaterials (NMs) are being widely recommended for water and wastewater treatments due to their unique physical and chemical properties. Several studies reporting the different advantages of nanotechnology in the remediation of wastewaters are numerous in the literature, but limited research effort has been directed toward understanding the fate and potential impacts of the solid residuals produced after the application of such technologies. The treatment of wastewaters containing organic and inorganic contaminants with NMs will result in residues containing NMs, used for the treatment, and several chemicals bound to them. It is then imperative to assess the environmental safety of these wastes. The present work aimed at investigating the ecotoxicity of solid residues resulting from the treatment of three effluents (olive oil mill, kraft pulp mill, and mining drainage) with two ( $\text{TiO}_2$  and  $\text{Fe}_2\text{O}_3$ ) NMs. Since these residues will eventually accumulate in the sediment of the aquatic compartment, the invertebrate *Chironomus riparius* was selected as test organism and exposed to the residues. A 10-day static laboratory bioassay was performed by exposing first instar larvae to artificial sediment mixed with solid residues of nano- $\text{TiO}_2$  and nano- $\text{Fe}_2\text{O}_3$  resulting from the treatment of each effluent. The effect on percentage of survival and growth (measured body length and extrapolated from the width of the head capsule) was assessed. Results showed that the residues from the treatments nano- $\text{TiO}_2(1.0 \text{ g L}^{-1})/\text{H}_2\text{O}_2(0.5 \text{ M})$  and nano- $\text{Fe}_2\text{O}_3(1.0 \text{ g L}^{-1})/\text{H}_2\text{O}_2(1.0 \text{ M})$  from olive oil mill effluent and nano- $\text{Fe}_2\text{O}_3(0.75 \text{ g L}^{-1})/\text{H}_2\text{O}_2(10 \text{ mM})$  from kraft paper mill effluent exhibited lethal toxicity to *C. riparius* larvae, as the percentage of survival reached only a maximum of 60%. Only the exposure with residues from the treatment with nano- $\text{Fe}_2\text{O}_3(0.75 \text{ g L}^{-1})/\text{H}_2\text{O}_2(10 \text{ mM})$  applied to the kraft paper mill effluent significantly affected the growth rate extrapolated from the head capsule width. In terms of growth rate computed through the measures of body length, it decreased significantly after exposure to the residues from the treatments

nano-TiO<sub>2</sub> (1.0 g L<sup>-1</sup>) and nano-Fe<sub>2</sub>O<sub>3</sub>(0.75 g L<sup>-1</sup>)/H<sub>2</sub>O<sub>2</sub>(10 mM) of kraft paper mill effluent and nano-Fe<sub>2</sub>O<sub>3</sub>(1.0 g L<sup>-1</sup>)/H<sub>2</sub>O<sub>2</sub>(1.0 M) of olive oil mill effluent. According to our study the solid residues resulting from the treatment of different effluents with NMs can promote negative effects on *C. riparius*. However, the effects are dependent on the type of effluent treated as well on the organic and inorganic compounds attached to the NMs.

**Keywords:** Chironomus riparius; sediment toxicity assessment; wastewater treatment; sludge; adsorbed compounds; nanoparticles.

## 6.1 Introduction

Surface and ground water resources are continuously facing profound changes and quality deterioration, caused by several anthropogenic activities. Industrial activities in particular are often responsible for the direct discharge of effluents containing organic pollutants and metals into aquatic ecosystems deteriorating the quality of water (Kanu and Achi, 2011). In an effort to combat the problem of water pollution the research for new wastewater treatment methodologies are increasing worldwide. In the last few years nanotechnology has become a hot topic for application in water and wastewater treatment due to the nanosized materials with their unique properties (Mohmood et al., 2013). The physical and chemical properties of nanomaterials (NMs) such as high surface area for adsorption, high activity for photocatalysis, antimicrobial properties for disinfection, magnetism, or other unique optical and electric properties make them excellent candidates for wastewater treatment (Qu et al., 2013). A plethora of reports have been published reporting the efficient application of nanoparticles for water and wastewater treatment purposes (Bora and Dutta, 2014; Hua et al., 2012; Xu et al., 2012), also demonstrated in our previous studies described in chapters III and V. However, the use of NMs to treat wastewaters will inevitably result in the production of sludge containing NMs and several other chemicals bound to them. Further some NMs may persist in suspension, which can ultimately be release into the aquatic compartment and thereby pose risks to living organisms. But to the best of our knowledge, no data about the fate and potential impacts of the solid wastes produced during the wastewater treatment exists

in previous reports. There is an important knowledge gap on the assessment of the risks associated with this type of residuals to the ecosystem, in particular to the sediments compartment that needs to be addressed.

In aquatic ecosystems, sediments play an important role, as they represent the ultimate sink for several contaminants (organic and inorganic) entering into water resources (Bettinetti et al., 2012). Moreover, the concentration of contaminants in sediments could be several orders of magnitude higher than in the overlying water. Sediments also provides a habitat and food source for a large number of benthic organisms, which themselves are important links in food chain web (Bettinetti et al., 2012). Chironomids represent a large fraction of benthic communities, being one of the most ubiquitous freshwater benthic invertebrates with a worldwide distribution and ecologically relevant to the aquatic food chain (Galluba et al., 2012; Hale et al., 2014). The larvae of the midge *Chironomus riparius* is widely employed as test organism to evaluate sediment toxicity (OECD, 2004; USEPA, 2000). Since *C. riparius* are sediment-dwelling and deposit-feeding organisms, they have a high probability of bioaccumulating high concentrations of contaminants through sediment ingestion (Oberholster et al., 2011), including contaminants like nanoparticles. Although in the last few years important reports were released concerning the ecotoxicological effects of NMs to benthic fauna (Bour et al., 2014; Nair et al., 2013; Oberholster et al., 2011; Waissi-Leinonen et al., 2012), the knowledge is still scarce and it needs to be addressed. However, to the best of our knowledge there is no report in the literature about the ecotoxicological effect of NMs bound to contaminants after the treatment of real hazardous organic and inorganic effluents to the *C. riparius*.

The objective of this study was to investigate the extent to which the solid residuals (SRs) produced during the treatment of three effluents (olive oil mill, kraft pulp mill, and mining drainage) with two ( $\text{TiO}_2$  and  $\text{Fe}_2\text{O}_3$ ) NMs impacts the survival and growth of *C. riparius*. The SRs used in this study resulted from previous treatments applied in chapters III and V and were chosen from the treatments that showed best performances in ameliorating the chemical characteristics and the toxicity of each effluent. For this purpose, the OECD standard method 218 (OECD, 2004) was used to test short-term effects on *C. riparius* (survival and growth) by exposing first instar larvae to artificial sediment spiked with solid residues containing nano- $\text{TiO}_2$  (239.6-

383.2 mg SR Kg<sup>-1</sup> d.w. of soil) and nano-Fe<sub>2</sub>O<sub>3</sub> (195.8-336.3 mg SR Kg<sup>-1</sup> d.w. of soil) attached to organic and inorganic compounds.

## **6.2 Materials and Methods**

### **6.2.1 *Chironomus riparius* sediment bioassay**

In this study, the SRs were directly mixed with the artificial sediment before the addition of the overlying water (as described in OECD (1984)) giving final concentrations of 195.8 to 383.2 mg SR kg<sup>-1</sup> soil (dry weight) depending on the treatment applied to the effluents (Table 1). The standard 10-day whole-sediment bioassay with *C. riparius* was carried out following the OECD (2004) and ASTM (2002) guidelines. The beakers were prepared 72 h prior to the beginning of the test with 150 g (wet weight) of the spiked sediment and 300 mL of reconstituted hard water (ASTM, 2002), to allow the sediment to attain the equilibrium (Oberholster et al., 2011). Three replicates were set up for each SR test consisting of ASTM hard water and the spiked sediment, while the control utilized ASTM and artificial sediment not spiked. Each replicate received 10 first instar *C. riparius* larvae, which were select at random and transferred to each beaker using a glass pipette, and after 30 min, aeration was started. The viability of the larvae was checked as they enter in the water column through their swimming action. The bioassays were conducted at 21±1°C, under a 16:8 h light dark photoperiod and water parameters (temperature, pH, conductivity and dissolved oxygen) were monitored in all beakers at the beginning and the end of the experiment. During exposure, the organisms were fed with 1 mg of Tetramin® per larvae every other day. After 10 days of exposure, all surviving larvae were gently removed, counted, and compared with the control. The growth rate (day<sup>-1</sup>) of *C. riparius* was also estimated by measuring the total body length and the head capsule size of each larva also at the beginning and at the end of the test.



Table VI.1- Mass ( $\text{mg Kg}^{-1}$ ) of the SRs containing nano-TiO<sub>2</sub> and nano-Fe<sub>2</sub>O<sub>3</sub> in the spiked sediment.

Effluent	Treatment	Mass ( $\text{mg Kg}^{-1}$ )
<b>Olive oil mill (OOMW)</b>	TiO <sub>2</sub> 1.0 g L <sup>-1</sup> / H <sub>2</sub> O <sub>2</sub> 0.5 M (Ti+HP 0.5)	356.90
	TiO <sub>2</sub> 1.0 g L <sup>-1</sup> / H <sub>2</sub> O <sub>2</sub> 1.0 M (Ti+HP 1.0)	361.47
	Fe <sub>2</sub> O <sub>3</sub> 1.0 g L <sup>-1</sup> / H <sub>2</sub> O <sub>2</sub> 0.5 M (Fe+HP 0.5)	335.70
	Fe <sub>2</sub> O <sub>3</sub> 1.0 g L <sup>-1</sup> / H <sub>2</sub> O <sub>2</sub> 1.0 M (Fe+HP 1.0)	336.33
<b>Kraft pulp mill</b>	TiO <sub>2</sub> 1.0 g L <sup>-1</sup> (TiO <sub>2</sub> 1.0)	325.40
	TiO <sub>2</sub> 0.75 g L <sup>-1</sup> (TiO <sub>2</sub> 0.75)	232.90
	TiO <sub>2</sub> 0.75 g L <sup>-1</sup> / H <sub>2</sub> O <sub>2</sub> 10 mM (Ti+HP 10)	242.30
	TiO <sub>2</sub> 0.75 g L <sup>-1</sup> / H <sub>2</sub> O <sub>2</sub> 75 mM (Ti+HP 75)	239.63
	Fe <sub>2</sub> O <sub>3</sub> 0.75 g L <sup>-1</sup> / H <sub>2</sub> O <sub>2</sub> 10 mM (Fe+HP 10)	218.43
	Fe <sub>2</sub> O <sub>3</sub> 0.75 g L <sup>-1</sup> / H <sub>2</sub> O <sub>2</sub> 75 mM (Fe+HP 75)	195.80
<b>Mining drainage</b>	TiO <sub>2</sub> 1.0 g L <sup>-1</sup> (TiO <sub>2</sub> 1.0)	383.23
	TiO <sub>2</sub> 0.75 g L <sup>-1</sup> (TiO <sub>2</sub> 0.75)	293.30
	Fe <sub>2</sub> O <sub>3</sub> 1.0 g L <sup>-1</sup> (Fe <sub>2</sub> O <sub>3</sub> 1.0)	334.13
	Fe <sub>2</sub> O <sub>3</sub> 0.75 g L <sup>-1</sup> (Fe <sub>2</sub> O <sub>3</sub> 0.75)	275.90

### 6.2.2 Characterization of the organic and inorganic content of SRs

The organic (e.g. organic compounds attached to nanoparticles) and inorganic content of SRs produced after the chemical treatment of OOMW, kraft pulp mill effluent and mining drainage were quantified in additional replicates obtained. The SRs were dried in an oven at 60°C until the weight was constant. A known amount of the dry of each SR was added to porcelain crucibles that were previously washed, sterilized in a muffle furnace for 30 min at 550°C, and weighted. To measure the amount of organic content the crucibles with SRs were placed in a muffle furnace at 550°C for 3 h, cooled at room temperature in a desiccators and weighed to the nearest 0.00001 mg. The difference between the oven-dry SR mass and the SR mass after combustion gave us the organic content. The inorganic content in the samples was then determined by heating at 1050°C for 4 h in the muffle furnace.

### 6.2.3 Statistical analyses

All statistical analyses were performed using the software SigmaPlot for Windows Version 11.0 (Systat Software, Inc., 2008). When the criteria of normality and equality of variances were met the data was analyzed using analysis of variance

(ANOVA). In alternative the nonparametric Kruskal-Wallis One Way ANOVA on ranks was performed whenever the criteria were not met. Significant differences in survival, development (head capsule width) and growth (body length increase) between the control and the treatments were identified using the Dunnett's test after ANOVA or the Dunns test after Kruskal-Wallis analysis.

### 6.3 Results

Figure 1 shows the percentages of survival of *C. riparius* under each treatment and the control. The results showed that the SRs from the treatments with nano-TiO<sub>2</sub>(1.0 g L<sup>-1</sup>)/H<sub>2</sub>O<sub>2</sub>(0.5 M) and nano-Fe<sub>2</sub>O<sub>3</sub>(1.0 g L<sup>-1</sup>)/H<sub>2</sub>O<sub>2</sub>(1.0 M) applied to the OOMW, and nano-Fe<sub>2</sub>O<sub>3</sub>(0.75 g L<sup>-1</sup>)/H<sub>2</sub>O<sub>2</sub>(10 mM) applied to the kraft paper mill effluent did exhibited lethal toxicity to *C. riparius* larvae compared to control, since the percentage of survival only reached 60% in some cases (Figure 1). In fact, the lowest percentage of surviving organisms was recorded for the sediments mixed with SR resulting from the treatments with nano-Fe<sub>2</sub>O<sub>3</sub>(1.0 g L<sup>-1</sup>)/H<sub>2</sub>O<sub>2</sub>(1.0 M) applied to the OOMW. In this case significant differences from the control were recorded (one-way ANOVA:  $F_{4,9}=4.492$ ;  $p=0.029$ ; Dunnett's test:  $p<0.005$ ). Although we did not recorded significant differences between the control and the SR from the kraft pulp mill effluent we recorded the same tendency of a higher mortality for the treatment with nano-Fe<sub>2</sub>O<sub>3</sub>(0.75 g L<sup>-1</sup>)/H<sub>2</sub>O<sub>2</sub>(10 mM).

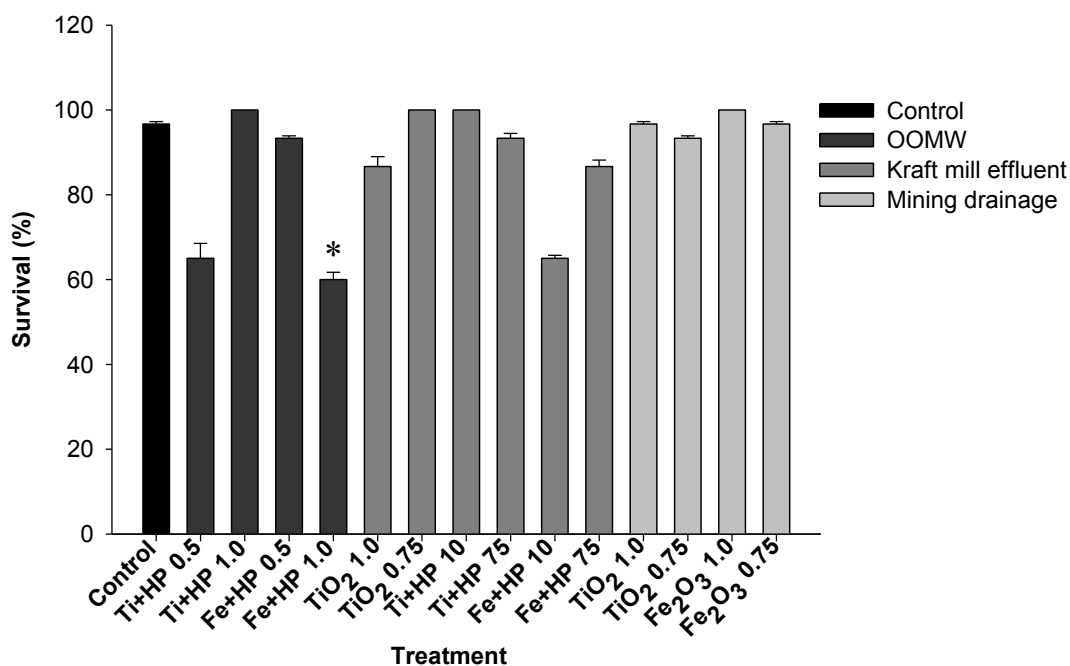


Figure VI.1- Percentages of survival of *C. riparius* larvae, following a 10-day exposure period, to control and sediments mixed with the SRs from the different effluent treatments. Asterisks indicate significant differences from the control ( $p < 0.05$ ).

Based on the larvae growth rate calculated from the head capsule width (Figure 2) significant differences were detected by the Kruskal–Wallis test followed by the Dunn’s test (d.f.=4;  $p<0.001$ ) between the control and the sediment mixed with SR from the treatment with nano-Fe<sub>2</sub>O<sub>3</sub>(0.75 g L<sup>-1</sup>)/H<sub>2</sub>O<sub>2</sub>(10 mM) applied to the kraft paper mill effluent (mean capsule width = 0.31±0.05 mm). Nonetheless, although we did not record significant differences in the mean head capsule width for the rest of the treatments, in some of them the larvae did not reached the four instar of development. According to Watts and Pascoe (2000) a *C. riparius* larvae with a head capsule width ranging from 0.29 mm to 0.45 mm, is a third instar larvae, while ranging from 0.49 mm to 0.63 mm, is a fourth instar larvae. The mean head capsule width of the surviving *C. riparius* larvae in the control was 0.49 mm (fourth instar larvae). For the sediment mixed with SRs from the treatments with nano-Fe<sub>2</sub>O<sub>3</sub>(1.0 g L<sup>-1</sup>)/H<sub>2</sub>O<sub>2</sub>(1.0M) of OOMW (mean head capsule with = 0.42±.010 mm), nano-TiO<sub>2</sub>(1.0 g L<sup>-1</sup>

<sup>1)</sup> of kraft paper mill effluent (mean head capsule with =  $0.45 \pm 0.11$  mm) and nano- $\text{TiO}_2$  ( $0.75 \text{ g L}^{-1}$ ) of the mining effluent (mean head capsule with =  $0.44 \pm 0.09$  mm) the *C. riparius* larvae did not reached the fourth instar.

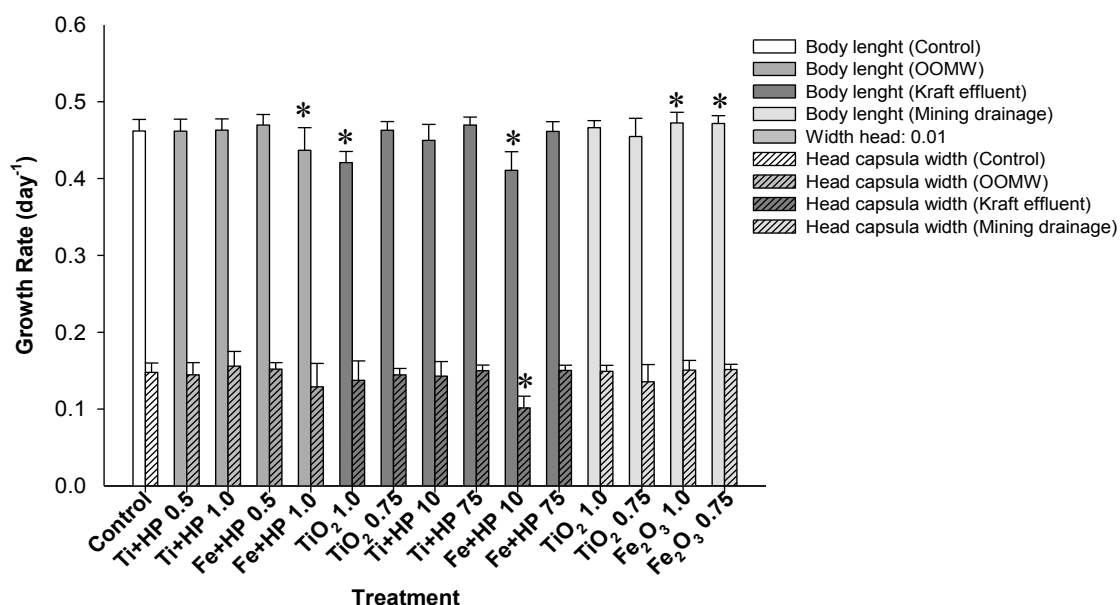


Figure V.2- Average growth rate of *C. riparius* in control and in sediment samples mixed with SRs from different effluent treatments, calculated based on body length and head capsule width of the larvae. Error bars represent the standard deviation. Asterisks indicate significant differences from the control ( $p < 0.05$ ).

In terms of growth rate computed based on the body length, the results in Figure 2 show a significantly decrease after the exposure to the SRs from the treatments with nano- $\text{Fe}_2\text{O}_3$  ( $1.0 \text{ g L}^{-1}$ )/ $\text{H}_2\text{O}_2$  ( $1.0 \text{ M}$ ) of OOMW (d.f.=4;  $p < 0.001$ ) and nano- $\text{TiO}_2$  ( $1.0 \text{ g L}^{-1}$ ) and nano- $\text{Fe}_2\text{O}_3$  ( $0.75 \text{ g L}^{-1}$ )/ $\text{H}_2\text{O}_2$  ( $10 \text{ mM}$ ) of kraft paper mill effluent (d.f.=6;  $p < 0.001$ ) when compared to the control. For the samples with SRs from the mining effluent treatments significant differences were also recorded for larvae growth in nano- $\text{Fe}_2\text{O}_3$  ( $1.0 \text{ g L}^{-1}$ ) and nano- $\text{Fe}_2\text{O}_3$  ( $0.75 \text{ g L}^{-1}$ ) treatments (d.f.=4;  $p < 0.001$ ), however the differences observed were due to a stimulatory effect in the growth of the larvae.

The organic and inorganic content of the solid residues generated by each treatment are presented in Table 2. The SRs resulting from the treatment of OOMW has a high content of organic compounds reaching values of  $0.142 \text{ mg}$  per  $\text{mg}$  of SR when nano- $\text{Fe}_2\text{O}_3$  ( $1.0 \text{ g L}^{-1}$ )/ $\text{H}_2\text{O}_2$  ( $0.5 \text{ M}$ ) was used, while the inorganic fraction, in the

maximum, reached values of 0.022 mg per mg of SR (Table 2). On the contrary, the SR resulting from the treatment of a mining effluent naturally presents higher content in inorganic compounds (Table 2), compared to the organic content. The treatments of the kraft pulp mill effluent give rise to SR with, in most of the cases, similar content in organic and inorganic compounds (Table 2).

Table VI.2- Organic and inorganic content (mg per mg of SR) of the residues formed after the treatment of OOMW, kraft paper mil effluent and mining effluent with nano-TiO<sub>2</sub> and nano-Fe<sub>2</sub>O<sub>3</sub>.

Effluent	Treatment	Organic compounds (mg/mg of SR)	Inorganic compounds (mg/mg of SR)
<b>Olive oil mill</b>	TiO <sub>2</sub> 1.0 g L <sup>-1</sup> / H <sub>2</sub> O <sub>2</sub> 0.5 M (Ti+HP 0.5)	0.107	0.012
	TiO <sub>2</sub> 1.0 g L <sup>-1</sup> / H <sub>2</sub> O <sub>2</sub> 1.0 M (Ti+HP 1.0)	0.128	0.022
	Fe <sub>2</sub> O <sub>3</sub> 1.0 g L <sup>-1</sup> / H <sub>2</sub> O <sub>2</sub> 0.5 M (Fe+HP 0.5)	0.142	0.015
	Fe <sub>2</sub> O <sub>3</sub> 1.0 g L <sup>-1</sup> / H <sub>2</sub> O <sub>2</sub> 1.0 M (Fe+HP 1.0)	0.129	0.000
<b>Kraft pulp mill</b>	TiO <sub>2</sub> 1.0 g L <sup>-1</sup> (TiO <sub>2</sub> 1.0)	0.079	0.030
	TiO <sub>2</sub> 0.75 g L <sup>-1</sup> (TiO <sub>2</sub> 0.75)	0.064	0.006
	TiO <sub>2</sub> 0.75 g L <sup>-1</sup> / H <sub>2</sub> O <sub>2</sub> 10 mM (Ti+HP 10)	0.049	0.029
	TiO <sub>2</sub> 0.75 g L <sup>-1</sup> / H <sub>2</sub> O <sub>2</sub> 75 mM (Ti+HP 75)	0.034	0.043
	Fe <sub>2</sub> O <sub>3</sub> 0.75 g L <sup>-1</sup> / H <sub>2</sub> O <sub>2</sub> 10 mM (Fe+HP 10)	0.033	0.031
	Fe <sub>2</sub> O <sub>3</sub> 0.75 g L <sup>-1</sup> / H <sub>2</sub> O <sub>2</sub> 75 mM (Fe+HP 75)	0.024	0.037
	TiO <sub>2</sub> 1.0 g L <sup>-1</sup> (TiO <sub>2</sub> 1.0)	0.033	0.086
	TiO <sub>2</sub> 0.75 g L <sup>-1</sup> (TiO <sub>2</sub> 0.75)	0.038	0.085
<b>Mining drainage</b>	Fe <sub>2</sub> O <sub>3</sub> 1.0 g L <sup>-1</sup> (Fe <sub>2</sub> O <sub>3</sub> 1.0)	0.022	0.052
	Fe <sub>2</sub> O <sub>3</sub> 0.75 g L <sup>-1</sup> (Fe <sub>2</sub> O <sub>3</sub> 0.75)	0.009	0.079

## 6.4 Discussion

In recent years, the application of NMs in the remediation of wastewaters has been increasing, showing great potential in the treatment of organic and inorganic effluents. However, the impacts of the sludge and of SRs produced during the treatment of wastewaters, which can be release to the aquatic ecosystem, have been neglected and rarely investigated. In order to understand the impacts of these solid wastes containing NMs and several other chemicals bound to them on the freshwater ecosystem, the sediment-dwelling *C. riparius* was exposed to the SRs resulting from several treatments. The SRs were generated in the treatment of organic and inorganic

effluents with nano-TiO<sub>2</sub> or nano-Fe<sub>2</sub>O<sub>3</sub>. In this study, the survival results showed a negative effect of the SRs from the nano-Fe<sub>2</sub>O<sub>3</sub>(1.0 g L<sup>-1</sup>)/H<sub>2</sub>O<sub>2</sub>(1.0M), and the nano-TiO<sub>2</sub>(1.0 g L<sup>-1</sup>)/H<sub>2</sub>O<sub>2</sub>(0.5M) treatments applied to the OOMW and from the nano-Fe<sub>2</sub>O<sub>3</sub>(0.75 g L<sup>-1</sup>)/H<sub>2</sub>O<sub>2</sub>(10 mM) treatment of the kraft pulp mill effluent (Figure 1). In fact, the SR coming from the treatment of OOMW with nano-Fe<sub>2</sub>O<sub>3</sub>(1.0 g L<sup>-1</sup>)/H<sub>2</sub>O<sub>2</sub>(1.0 M) also caused negative effects on the development of *C. riparius* affecting significantly the body length, and although not significantly, the instar of the larvae (Figure 2). One of the few studies addressing the toxicity of NMs in *Chironomus* larvae is the study of Oberholster et al. (2011). These authors conducted a study to determine the effect of seven NMs, including nano-Fe<sub>2</sub>O<sub>3</sub> (size: 50-150 nm), on survival, growth, enzyme activities and DNA fragmentation of *C. tentans*. At the highest concentration tested for nano-Fe<sub>2</sub>O<sub>3</sub> (5000 µg Kg<sup>-1</sup>) significant differences were recorded for all the parameters tested, including survival and growth length. In our treatment with nano-Fe<sub>2</sub>O<sub>3</sub>(1.0 g L<sup>-1</sup>)/H<sub>2</sub>O<sub>2</sub>(1.0M), although we used nano-Fe<sub>2</sub>O<sub>3</sub> and negative effects were recorded (Figure 1 and 2), we cannot assume that these effects were only due to the NM. Some reports already shown that OOMW is highly toxic to benthic organisms, such as *Daphnia magna*, *Gammarus pulex* and *Hydropsyche peristerica* (Justino et al., 2009; Karaouzas and Cotou, 2011). So, these effects can have been somehow related to the toxic organic compounds present in the OOMW that were adsorbed to the NM. However, other treatments using nano-Fe<sub>2</sub>O<sub>3</sub> in the system [nano-Fe<sub>2</sub>O<sub>3</sub>(1.0 g L<sup>-1</sup>)/H<sub>2</sub>O<sub>2</sub>(0.5 M) also applied to the OOMW, which in fact adsorbed more organic compounds (Table 2), but a lower concentration of H<sub>2</sub>O<sub>2</sub> was used, no negative effects were recorded. Some authors have reported that, although the great efficiency of the photocatalytic process, in some cases there is the formation of secondary compounds during the treatment of the effluent, mainly due to the partial oxidation of organic compounds, more toxic than the initial ones (Oller et al., 2011; Pereira et al., 2009; Rizzo, 2011). The organic compounds adsorbed to nano-Fe<sub>2</sub>O<sub>3</sub> in the SR of the treatment nano-Fe<sub>2</sub>O<sub>3</sub>(1.0 g L<sup>-1</sup>)/H<sub>2</sub>O<sub>2</sub>(1.0M) were probably more toxic compared to the other treatments with nano-Fe<sub>2</sub>O<sub>3</sub> for OOMW. The same has likely happened in the sediment mixed with the SR from the treatment of kraft pulp mill with nano-Fe<sub>2</sub>O<sub>3</sub>(0.75 g L<sup>-1</sup>)/H<sub>2</sub>O<sub>2</sub>(10 mM). All the endpoints assessed, survival, body length and head capsule width of *C. riparius* larvae were negatively affected (Figure 1 and 2).

Regarding the SRs containing nano-TiO<sub>2</sub> we only recorded a negative effect in the treatment using nano-TiO<sub>2</sub> (1.0 g L<sup>-1</sup>) applied to the kraft pulp mill effluent (Figure 2). However, the findings of Lee et al. (2009) demonstrated that 1 mg L<sup>-1</sup> of nano-TiO<sub>2</sub> (7 and 20 nm) did not affect reproduction, growth, or mortality of the aquatic midge *C. riparius*. Anyway, in our study the SRs (with nano-TiO<sub>2</sub>) were directly mixed in the sediment, while the exposure route in the study of Lee et al. (2009) the nanoparticles were dispersed with a sonicator in the culture medium. During its larval stage *C. riparius* resides within the sediment being directly exposed to the contaminants, and in addition, these organisms can ingest sediment particles during the feeding process (Bour et al., 2014; Oberholster et al., 2011). So the ingestion of the SRs containing nano-TiO<sub>2</sub> nanoparticles and the adsorbed organic compounds can probably occur promoting a negative effect on the growth and development of these organisms as recorded in our study. Also the concentration of nano-TiO<sub>2</sub> was more than 100 times higher in our study when compared with concentration tested by Lee et al. (2009). Nonetheless, the negative effects recorded can not be attributed only to nano-TiO<sub>2</sub>, since no other treatment with nano-TiO<sub>2</sub> affected *C. riparius*, however nano-TiO<sub>2</sub> (1.0 g L<sup>-1</sup>) was the treatment who recorded a higher amount of organic waste adsorbed from the kraft pulp mill effluent (Table 2). These effluents contain high amounts of toxic chemicals such as chlorinated compounds, tannins, resin acids and phenols, lignin and dioxins which can cause considerable damage to the organisms of the receiving waters (Ali and Sreekrishnan, 2001; Pereira et al., 2009). Kienle et al., (2013), for example, demonstrated that a pulp deposit mixed with sediment can be highly toxic and promote negative effects on the mortality, emergence and locomotor activity of *C. riparius*. Some reports have already shown that the toxicity of the NMs can not only be inherent to the particles themselves, but also be influenced by the interaction with toxic environmental pollutants (Museum, 2011), like the toxic compounds present in the kraft pulp mill effluent. For example, in the study of Zhu et al. (2011) no toxic effects to abalone embryos were recorded after exposure to 2 mg L<sup>-1</sup> of nano-TiO<sub>2</sub>. Yet the presence of the same concentration of nano-TiO<sub>2</sub> enhanced the negative effect of tributyltin (TBT) compared with TBT alone. The same effect was also present in the study of Hartmann et al. (2010). Three TiO<sub>2</sub> nanoparticles with different sizes (10, 30 and 300 nm) were responsible for the inhibition of algal growth. Further, the toxicity of

cadmium in the presence of  $2 \text{ mg L}^{-1}$  of  $\text{TiO}_2$  with 30 nm was enhanced when compared to the exposure to cadmium alone (Hartmann et al., 2010). From both studies we can conclude that NMs can function as carriers promoting the uptake and the internalization of pollutants by organisms, giving rise to the so called Trojan horse effect; or synergistic effects may occur between the NMs and the pollutants adsorbed to them (Limbach et al., 2007; Musee, 2011). This explanation can justify the results of our study for the toxicity recorded in the sediments mixed with the SR originated from the treatment with nano- $\text{TiO}_2$  ( $1.0 \text{ g L}^{-1}$ ) of the kraft pulp mill effluent as well in the case of the SR coming from nano- $\text{Fe}_2\text{O}_3$  ( $1.0 \text{ g L}^{-1}$ )/ $\text{H}_2\text{O}_2$  (1.0M) treatment applied to the OOMW.

As for the SRs resulting for the treatment of the mining drainage no toxic effects were observed on the survival and growth of *C. riparius* after 10 days of exposure. In opposition growth stimulation (body length) was observed with the sludge from the treatments with nano- $\text{Fe}_2\text{O}_3$  0.75 and  $1.0 \text{ g L}^{-1}$  (Figure 2). These results reinforce the idea that the toxic effects observed for the SRs of OOMW and pulp mill effluent are not due to the NMs, but probably due to organic compounds adsorbed to them. While the organic compounds present in OOMW and pulp mill effluent are generally responsible for negative effects (Justino et al., 2012; Pereira et al., 2009), the ingestion of metals and metalloids (including iron) present in the mining effluent can be even beneficial or toxic, depending on the concentration. There are several macronutrients (e.g. calcium, magnesium, potassium and sodium) and micronutrients (e.g. as chromium, cobalt, copper, iron, manganese, nickel, selenium and zinc) essential for the growth and survival of organisms (Chapman and Wang, 2000) and although at low levels these elements are essential, too much can also be lethal. If we look to our data both SRs with nano- $\text{Fe}_2\text{O}_3$  from the mining effluent treatments that promoted the growth stimulation were those with the lowest inorganic content residues adsorbed (Table 2). These results suggest that the concentration of the metals and metalloids adsorbed to the NMs in the SRs are above the essential limit but below the toxic boundary. A number of studies have shown that chironomid larvae are able to survive and even increase in number when exposed to environments contaminated with metals (De Bisthoven et al., 2005; Stuijzand et al., 2000). This tolerance to metals and



metalloids has been attributed to the ability of chironomid larvae to excrete or detoxify the excessive amount of these elements (Gillis and Wood, 2008).

## 6.5 Conclusion

Currently, the knowledge available about the potential toxicity of the residues formed after the treatment of organic and inorganic effluents with NMs is still very limited. So in this study the effect of the solid residues produced during the treatment of OOMW, kraft pulp mill effluent and mining effluent with nano-TiO<sub>2</sub> and nano-Fe<sub>2</sub>O<sub>3</sub> was assessed using the sediment-dwelling *C. riparius*. The aquatic midge was used since sediments play an important role in the aquatic system, functioning as sink for several anthropogenic contaminants, including effluents. In this study we demonstrated that certain solid wastes resulting from the treatment of different effluents with NMs, which can accidentally attain the freshwater ecosystems, have the potential to negatively affect *C. riparius* survival and growth, depending not only on the type of effluent treated but also on the organic or inorganic compounds bound to the NMs. The SR resulting from the treatment of mine drainage did not affect neither the survival nor at sub-lethal level, however, the same was not true for the OOMW and for the Kraft pulp mill effluent. Still, further studies are needed to determine in detail which organic and inorganic compounds were adsorbed by the NMS. The results of this study highlight the need for taking into consideration the overall consequences of wastewater treatments with NMs as they could give rise to SR with harmful effects to ecosystem health.

## 6.6 References

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# Chapter VII

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Conclusions and final remarks



## 7.1 Conclusions and final remarks

Increased industrial, mining and agricultural activities have resulted in the generation and accumulation of huge amounts of different pollutants as part of effluents, which are the main cause of the deterioration of aquatic systems (Lokhande et al., 2011). Depending on the activity, resulting wastewaters can contain high loads of organic and inorganic compounds, some of them with high toxicity and recalcitrant potential. Despite all the efforts that have been made trying to find suitable treatments for industrial effluents none of them were totally efficient, especially in terms of toxicity (Justino et al., 2012; Pereira et al., 2009). Hence, it is imperative to develop innovative technologies. Advances in nanotechnology offer leapfrogging opportunities to treat pollutants in water and soil. Nanotechnology holds not only the promise of enhance the existing water and wastewater treatments, but also develop new processes. The unique proprieties of nanomaterials such as strong adsorption, enhanced redox and photocatalytic proprieties, makes them excellent candidates for the treatment of industrial effluents (Mohmood et al., 2013; Qu et al., 2013, 2012). However, the exponentially growth of publications about the application of nanomaterials in wastewater treatment has not been accompanied by knowledge concerning the ecotoxic effects of NMs once used and released into the environment. More important is the ecotoxicological assessment prior application on wastewater treatment, intended in preventing the intentional introduction of toxic nanomaterials in the environment. The present work aims to contribute for the increase of knowledge about the potential effects of NMs on the environment, especially in the aquatic compartment, and also give new insights for using nanoparticles in the treatment of complex industrial wastewaters.

Take this in consideration, our first approach aimed to obtain a comprehensive effect assessment of four nanomaterials with respect to aquatic organisms. Both nickel oxide were the most toxic for all the freshwater species, however, contrary to what would be expected, the NiO with lower size did not promote the highest toxicity. *Daphnia magna* was the most sensitive organism recording the lowest EC<sub>50</sub> and LOECs values for both nano-NiO and nano-TiO<sub>2</sub>. This could be explained mainly because they

are filter feeders and were likely exposed to a large number of nanoplastics (NPs) and NMs-aggregates over the exposure time. Nevertheless, marine species were not acutely affected by any of the NPs tested in the range of concentration presented. Our results show that, in biological environments, the toxic effects of NPs will depend on the physical and chemical parameters of the receiving medium, as it can contribute for the agglomeration, solubility and surface charge of NPs, making difficult to predict their environmental fate and ability to promote toxic effects. Although no apparent relationship was found between surface charge, zeta potential, size and toxicity for the organisms tested, the particle chemical composition and solubility played a key role. From our data,  $\text{Fe}_2\text{O}_3$  and  $\text{TiO}_2$  NMs have shown to be the ones with less environmental risk, and hence those more appropriate for a safe use in wastewater treatment.

Considering the previous results the two NMs with less risk were applied in the treatment of a highly toxic agroindustrial effluent, the olive oil mill wastewater (OOMW). The use of metallic oxide nanoparticles has been suggested, but few data exists demonstrating their real effectiveness in the treatment of wastewaters with a high organic load. The application of advanced oxidation processes (AOPs) has showed great efficiency for wastewaters containing high chemical stability and/or low biodegradability like OMW (Oller et al., 2011; Poyatos et al., 2010). The use of NMs as catalysts can increase even more the efficiency of advanced oxidation processes due to its enhanced photocatalytic properties. The application of NMs and hydrogen peroxide greatly increased the photodegradation process compared to the systems with only nano- $\text{TiO}_2$ /UV and nano- $\text{Fe}_2\text{O}_3$ /UV for the physic-chemical parameters. However, the performance of the treatment is a function of operation conditions such as catalyst type and loading, as well the presence or not of an oxidant and its corresponding concentration. Another question was raised with this chapter (III), the importance of an ecotoxicological evaluation before and after wastewater treatment. Although there are several reports showing the application of advanced oxidation processes for wastewater treatment the majority of the studies are not followed by an ecotoxicological assessment to validate its effectiveness in terms of toxicity reduction



(Justino et al., 2012; Pereira et al., 2009). So in this study an assessment of the toxicity before and after OMW treatment was done, showing that although we recorded higher reductions in the physic-chemical parameters for the treatments using NMs and  $H_2O_2$  this was not followed by the higher decrease in toxicity to the bacteria *V. fischeri*. On the contrary the treatment with the most significant reductions in this parameter was the  $H_2O_2$ /UV system. Although the application of advanced oxidation processes can be easily applied with great results, sometimes this type of treatment may lead to an increase in effluent's toxicity. Trying to ameliorate the toxicity of the effluent in the chapter IV we used a sequential combination of treatments using photocatalytic processes with nano- $TiO_2$  or nano- $Fe_2O_3$  and biological treatment with two species of fungi (*Phanerochaete chrysosporium* and *Pleurotus sajor caju*). Although the application of biological processes are the cheapest and most environmentally friendly solution it not always provide satisfactory results (Hanafi et al., 2010). Mainly due to the toxic compounds present in the wastewaters the treatment with biological processes usually requires a dilution step. To overcome these drawbacks, the application of a pre-treatment is a feasible option, reducing or even eliminating the antimicrobial compounds of the wastewater. This combination of processes showed good results in removing COD, total phenolic content and toxicity, however, in terms of colour the biological treatment was not able of promoting any reductions. The most promising methodology taking into consideration the integrity of the receiving ecosystem is the combined application of the pre-treatment with nano- $Fe_2O_3$ /UV and *P. chrysosporium*. A single-stage chemical or biological treatment is unlikely to achieve satisfactory results at reasonable cost so a sequential treatment using both may be the optimum solution for OOMW.

Besides olive oil mill wastewater, the efficiency of metallic oxide nanomaterials was also addressed in other concerning wastewaters, the kraft pulp mill effluent and mining drainage. Both effluents are known to cause severe effects on the aquatic compartment (Pereira et al., 2009; Sarmiento et al., 2011). The operation conditions have a main effect on the efficiency of the photocatalytic process in the organic effluent. The best result was achieved at pH 3 and once again using the combination of

nanomaterials and hydrogen peroxide. This is expected, since both catalyst and  $H_2O_2$  produce hydroxyl radicals (Ahmed et al., 2010). However, for the inorganic effluent the most efficient treatments were the ones only with nanomaterials, and although only three out of seven metals and metalloids were reduced, higher reductions in terms of toxicity were achieved. This fact may be attributed to the photocatalytic reduction of the metals and metalloids promoted by the nanomaterials, making them less available to uptake by organisms.

Preliminary results showed that nanomaterials can be applied to wastewater treatment with promising results, however more detailed studies would be necessary to determine the optimal conditions for practical use on an industrial scale. Although the advantages of nanotechnology in the remediation of wastewaters have been recognized, most of the research lacks an ecotoxicological evaluation in all the process, especially for the solid residuals produced after the application of such technologies. These residuals, which contain NMs and several other chemicals bound to them (organic and inorganic compounds), can accidentally reach the aquatic compartment and accumulate in the sediment. In the present work, the environmental safety of solid residuals resulting from the treatment of the three effluents (olive oil mill, kraft pulp mill, and mining drainage) was examined. The results suggest that these solid residues can in fact negatively affect *C. riparius*, however the effects depend on the effluent that was treated but also on the compounds bound to the NMs. The work carried out in this thesis highlights the need for a full ecotoxicological evaluation at different steps of the process when using nanomaterials for wastewater treatment.

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