**Universidade de Aveiro** Departamento de Biologia
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**MÁRCIA LUÍSA BESSA DA SILVA**

# Avaliação do Impacto Ecológico de Contaminantes

# Evaluation of the Ecological Impact of Contaminants

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# Evaluation of the Ecological Impact of Contaminants

Tese apresentada à Universidade de Aveiro para cumprimento dos requisitos necessários à obtenção do grau de Doutor em Biologia, realizada sob a orientação científica do Doutor Fernando Gonçalves, Professor Associado com Agregação do Departamento de Biologia da Universidade de Aveiro, da Doutora Ruth Pereira, Professora Auxiliar com Agregação do Departamento de Biologia da Faculdade de Ciências da Universidade do Porto e do Doutor João Carlos Marques, Professor Catedrático do Departamento de Ciência da Vida da Faculdade de Ciências e Tecnologia da Universidade de Coimbra

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*Aos meus Pais, José e Isaura,*

*pelas nossas conquistas...*

**o júri**

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**palavras-chave**

ecossistemas de água doce de baixa profundidade, atividades agrícolas, poluição difusa, eutroficação, pesticidas, relações tróficas, avaliação ecotoxicológica, análise de risco, nanomateriais, remediação

**resumo**

As atividades agrícolas são uma das principais fontes de poluição para os ambientes circundantes. A poluição difusa com nutrientes, pesticidas, metais [ou produtos de proteção de plantas (PPPs) em geral] foi identificada como uma das principais ameaças para os recursos de águas superficiais e subterrâneas. De facto, principalmente através da lixiviação e escoamento dos campos agrícolas adjacentes, os nutrientes e contaminantes podem entrar nas massas de água promovendo a ocorrência de blooms de algas e o aumento da eutroficação do sistema. Os processos de eutroficação são causados pelo aumento direto da concentração de nutrientes, mas também pela incapacidade das populações de consumidores primários em controlar a proliferação de microalgas devido aos impactos diretos ou indiretos dos PPPs. Nestes cenários de poluição difusa, e para se ter uma boa perspectiva de como o sistema funciona, é também necessário entender as interações entre os compartimentos aquático e terrestre para avaliar a contribuição dos solos da bacia de drenagem, com PPPs, para os lagos pouco profundos. Neste contexto, o presente estudo contribuiu para melhorar o conhecimento sobre os efeitos da poluição difusa, em particular sobre a forma como a exposição ao herbicida pendimetalina, sob concentrações ambientalmente relevantes, pode comprometer a qualidade nutricional do fitoplâncton, afectando indiretamente a sustentabilidade das populações de consumidores primários dentro das cadeias tróficas de água doce. Além disso, este estudo apresentou uma adaptação de Análise de Risco Ecológico (ARE), previamente proposto para locais contaminados, e demonstrou a sua aplicação numa área de poluição difusa por atividades agrícolas, na bacia de drenagem de uma lagoa pouco profunda, de água doce, no centro de Portugal (Lagoa da Vela, Figueira da Foz). Assim, combinando análises ecotoxicológicas e químicas para um rastreio dos solos circundantes, os riscos foram calculados para o primeiro nível do esquema da ARE. Por fim, o nosso estudo teve também como objetivo avaliar a aplicabilidade de uma abordagem baseada em nanotecnologia para a remediação de massas de água interiores pouco profundas expostas a poluição difusa. De acordo com os nossos resultados, a qualidade nutricional das algas expostas ao herbicida pendimetalina (Prowl®) revelou uma ligeira diminuição dos níveis de ácidos gordos e um aumento no seu conteúdo em proteínas, enquanto não foram registadas diferenças significativas nos hidratos de carbono. A reprodução de *Daphnia magna* foi significativamente reduzida em organismos alimentados com microalgas previamente expostas ao herbicida, embora nenhum efeito significativo tenha sido registado no tempo de ocorrência da primeira ninhada, no tamanho geral das ninhadas e na taxa de crescimento. Adicionalmente, foi registado um conteúdo corporal de pendimetalina de 4.226 $\mu g\ g^{-1}$ em *D. magna* alimentada com algas expostas ao pesticida. Não foi possível estabelecer uma relação de causa e efeito direta entre os efeitos registados nos dafnídeos e a qualidade nutritiva das microalgas. Os efeitos tóxicos do pesticida transferido das microalgas para os consumidores podem também ter sido responsáveis pelos efeitos sobre a reprodução dos organismos. Utilizando uma bateria de bioensaios efetiva em termos de tempo e custos, demonstrou-se que o início da fase de rastreio da ARE (nível 1), com

**resumo (cont.)**

a aplicação da Linha de Evidência Ecotoxicológica (do inglês: *EcotoxLoE*), foi informativa para um primeiro rastreio dos solos da bacia de drenagem da lagoa da Vela que podem representar riscos mais elevados para a lagoa. A Linha de Evidência Química (do inglês: *ChemLoE*), aplicada já apenas a um número reduzido de solos, reforçou as conclusões baseadas na *EcotoxLoE*, destacando o papel dos organismos do solo como indicadores mais sensíveis dos efeitos das misturas de contaminantes presentes nesta matriz. Finalmente, a aplicação de nanopartículas de $TiO_2$ na remediação de massas de água mostrou-se eficaz no controlo da concentração interna de nutrientes e na eliminação de blooms algais. No entanto, esta abordagem não foi inócua para as espécies não-alvo de sistemas eutróficos, como foi testado em sistema de microcosmos multiespecífico. Assim, estudos futuros devem ser focados em avaliar a eficácia de diferentes nanomateriais, inócuos e ambientalmente sustentáveis, para a prevenção e mitigação blooms de algas em lagos de baixa profundida através de uma abordagem integrada, semelhante à testada no nosso estudo.

**keywords**

shallow freshwater ecosystems, agricultural activities, diffuse pollution, eutrophication, pesticides, trophic-chain relationships, ecotoxicological evaluation, risk assessment, nanomaterials, remediation

**abstract**

Agricultural activities are one of the major sources of pollution in the surrounding environments. Diffuse pollution with nutrients, pesticides and metals [or plant protection products (PPPs) in general] has been identified as one of the most critical threats to both surface and groundwater resources. In fact, mainly through leaching and runoff from adjacent agricultural fields, the nutrients and contaminants can enter the water bodies promoting the occurrence of algal blooms and an increased eutrophication of the system. The eutrophication processes thus may be caused by the direct increase in nutrient loads, but also by the inability of primary consumers in controlling the blooms due to direct or indirect impacts of PPPs on their populations. In these scenarios of diffuse pollution, and in order to have a good overview on how the system works, is also necessary to understand the interactions between aquatic and terrestrial compartments to assess the contribution of the soils of the drainage basin, with PPPs, to shallow freshwater lakes. In this context, the present study contributed to increase knowledge about the effects of diffuse pollution, particularly how the exposure to the herbicide pendimethalin, under environmentally relevant concentrations, may compromise the nutritional quality of phytoplankton, thus indirectly affecting the sustainability of primary consumers' populations within freshwater food webs. Furthermore, this study presented an adapted ecological risk assessment (ERA) framework, previously proposed for contaminated sites, and demonstrated its application on an area undergoing diffuse pollution from agricultural activities, in the drainage basin of a shallow freshwater lake in the Center of Portugal (Lake Vela, Figueira da Foz). Thus, combining ecotoxicological and chemical analysis for a soil screening assessment, risks were calculated for a first tier of the ERA framework. Finally, it was also aimed to assess the application of a nanotechnology-based approach for the remediation of shallow freshwater bodies exposed to diffuse pollution. According to our results, the nutritional quality of algae exposed to the herbicide pendimethalin (Prowl®) revealed a slight decrease in fatty acids levels and an increase of protein content, while no significant differences were recorded for carbohydrates. The reproduction of *Daphnia magna* was significantly reduced in organisms fed with algae previously exposed to the herbicide, but no significant effects were recorded at the time to first broad, brood size and growth rate. Additionally, an internal pendimethalin body burden of 4.226 $\mu$g $g^{-1}$ was recorded on *D. magna* fed with algae exposed to the pesticide. It was not possible to establish a direct cause-effect relationship between the effects recorded on daphnids and the nutritional quality of microalgae. The toxic effects of the pesticide transferred from the algae to consumers may also have been responsible for the effects on organism's reproduction. Using a cost-effective and time-effective battery of bioassays, the start of the ERA framework (tier 1), with the application of the Ecotoxicological Line of Evidence (EcoToxLoE), was informative for a first screening of soils from the Lake Vela drainage basin that may represent higher risks to the lake. The Chemical Line of Evidence (ChemLoE), only applied to a reduced number of soils, reinforced the conclusions made based on the

**abstract (cont.)**

EcoToxLoE, highlighting the role of the soil organisms as more sensitive indicators of the effects of mixtures of soil contaminants. Finally, the application of $TiO_2$ nanoparticles on water remediation approaches proved to be effective in controlling the internal loading of nutrients and for eliminating algal blooms. Nevertheless, this approach was not innocuous for non-target species of eutrophic systems, as tested under microcosm multispecies test systems. Thus, future efforts should be focused on testing different nanomaterials, both innocuous and environmentally sustainable, for their effectiveness in preventing or mitigating algal blooms in shallow lakes through an integrated approach, similar to the one tested in our study.

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*Além da conversa das mulheres, são os sonhos que seguram o mundo na sua órbita. Mas também são os sonhos que lhe fazem uma coroa de luas, por isso o céu é o resplendor que há dentro da cabeça dos homens, se não é a cabeça dos homens o próprio e único céu.*

JOSÉ SARAMAGO, *Memorial do Convento*

# Chapter I

## General Introduction

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Bessa da Silva, M., Gonçalves, F., Pereira, R. Portuguese shallow eutrophic lakes: Evaluation under the Water Framework Directive and possible physicochemical restoration measures.

# General Introduction

## 1.1. Shallow freshwater lakes coastal dynamics and its major threats

The Millennium Ecosystem Assessment (Millenium Ecosystem Assessment, 2005) gave the world several important insights about the dependence of human society from natural resources, contextualizing human-environmental interactions and clarifying the impacts of human activities on ecosystems, and their feed-back in terms of human health and well-being caused by the loss of important ecosystem services.

Small water bodies (i.e. ponds, small lakes, low-order streams, springs) are globally the most numerous freshwater environments and most of water-related ecosystems are regulated by them (Biggs et al., 2017). In the European context, freshwater ecosystems include pure aquatic ecosystems, such as rivers, lakes, wetlands and floodplains (Harrison et al., 2010), and these systems are critical for freshwater biodiversity in the provision of many services for human welfare and also for ecosystem services (Jogo and Hassan, 2010; López-Merino et al., 2011; Namaalwa et al., 2013). Particularly related to services delivery, inland and coastal aquatic resources are vital net carbon sinks mediating carbon cycling (Brett et al., 2017; McDonald et al., 2012a; Sheng et al., 2004), they play a role in water retention and regulation, balancing the hydrography during low-flow conditions, and also provide a key contribution to climate regulation (Harrison et al., 2010; Millenium Ecosystem Assessment, 2005). But over time, these freshwater ecosystems become fragile and vulnerable as a result of their exposure to several anthropogenic stressors (Callahan et al., 2017; Kotze et al., 2012). The rapid human population growth in the 1980s accelerated the eutrophication of shallow aquatic environments, not only by altering the natural cycles of carbon, nitrogen and phosphorus (Hines et al., 2013; McDonald et al., 2012; Zamparas and Zacharias, 2014), but also by promoting the entrance of nutrients in aquatic systems (Pereira et al., 2010; Schindler, 2012). Despite the relevance of direct input of nutrients in these aquatic systems by the municipal and industrial sewage discharges, the nonpoint sources are generically of a greater relevance, due to their larger incidence, extension and control difficulties (Howarth et al., 2000). Runoff of manure, biosolids, fertilisers and/or plant protection products (PPPs) intentionally applied to agricultural soils are considered the major

contributors of nutrients and of contaminants in the adjacent water bodies (Hijosa-Valsero et al., 2016; Horton et al., 2017; Schmidt Rivera et al., 2017; Shappell et al., 2016). Moreover, some of these water bodies like shallow lakes, for example, present low depths and low water inflow rates, which reduces their dilution capacity and enhance the sediment retention of nutrients and/or contaminants (Abrantes et al., 2006b; Bazzanti et al., 2017; Janse et al., 2008; Liu et al., 2016; Xu et al., 2016). Generically, the nutrient enrichment in shallow lakes in the presence of some environmental/physical conditions, such as warm temperatures, low turbulence, high light intensity/radiation, and low grazing rates stimulates the increase of primary production in lakes (Darchambeau et al., 2014; Kauer et al., 2015; LaBuhn and Klump, 2016). It is well known that these factors, alone or combined, can cause severe co-lateral problems, such as the occurrence of cyanobacteria blooms (Berry et al., 2017; Lee et al., 2017; Paerl and Otten, 2013; Scholz et al., 2017), as well as several indirect effects that disrupt natural communities. Cyanobacteria blooms are characterized by a decrease in the biodiversity of aquatic ecosystems, resulting from the dominance of one or a few species of phytoplankton, which upsets the ecosystem balance (Grabowska et al., 2013). The most well-known species are from the genera *Anabaena* and *Microcystis* and generally they produced different types of toxins in freshwater systems worldwide (Dai et al., 2008; Hautala et al., 2013; Lee et al., 2017b; Oberholster et al., 2009; Tonk et al., 2009). These toxins are predominantly within cyanobacterial cells, but their concentration may increase into a waterbody after the decay of blooms and the subsequent cell's lysis (Mucci et al., 2017; Zamyadi et al., 2012), potentially endangering animals and plants (Carmichael and Boyer, 2016; Oberholster et al., 2009; Sotton et al., 2017). Furthermore, the senescence of algal blooms consequently stimulates the deposition and accumulation of organic matter into the benthic zone (Nyenje et al., 2010). This process involves oxygen consumption and the development of hypoxic conditions with consequences for the aquatic environments, particularly to the bottom dwelling organisms, as macroinvertebrates and crustaceans. Some sensitive fish species may escape, be immobilized or even collapse (Conley et al., 2009; Paerl and Otten, 2013; Scavia et al., 2014; Smith et al., 1999). In this context, eutrophication could directly alter the ecological integrity of biological benthic-pelagic

communities of lakes (Izmest'eva et al., 2016; Qin et al., 2006). During many years, it was assumed that nutrients bioavailability, light and temperature were the main factors regulating phytoplankton and their interactions with consumers in the sustainability of the food webs (Arhonditsis et al., 2004; Arhonditsis and Brett, 2005). In fact, the food webs were primarily regulated by bottom-up controls, with phytoplankton acting as an energy source that flowed through the food webs in limnetic systems (Gliwicz, 2002; Jeppesen et al., 2003). However, other researches had consistently showed that consumers also regulated food webs, in which zooplankton is controlled by fish grazing, and in turn phytoplankton is controlled by zooplankton, via top-down regime (Horn and Horn, 2008; Ingrid et al., 1997; Jeppesen et al., 2003; Jürgens et al., 1999). Many years of research were necessary to understand that cascading trophic interactions and nutrient loading models should be seen as complementary, not contradictory (Mcqueen et al., 1989; Menge, 2000; Ripple et al., 2016). In this context, additional research in aquatic community regulation had combined bottom-up (i.e. food resources) and top-down (i.e. predation) effects to evaluate the abundance patterns among some functional groups, mainly those related to fleshy algal biomass, which is usually related to the eutrophic scenarios (Buskey, 2008; Ibáñez et al., 2012; Jiang et al., 2008; Shurin et al., 2012). Particularly in eutrophic shallow lakes, top-down control is more important than bottom-up effects, not only by the high concentrations of edible phytoplankton, but also by the dominance of predation forces in regulating the abundance, biomass and species composition of fleshy algae (Jürgens et al., 1999). Furthermore, there are several evidences that top-down control is more important in shallow lakes than in deep lakes, since shallow lakes are easier to manipulate and lake restoration by means of biological manipulation seems more effective than in deep lakes (Jeppesen et al., 1997). Despite lake dynamics are dependent of several abiotic factors, such as temperature, turbidity, climate change, lake depth and lake residence time, the shallow waters are particularly susceptible to increases of nutrients supply (Laspidou et al., 2017). According to Jeppesen et al. (2003), the predator control is largely related to the total phosphorus (TP) available, and the cascading effect of herbivory pressure under phytoplankton decreases with the increase of TP. For that reason, phosphorus (P) and also nitrogen (N) are the two major

nutrients involved in the eutrophication phenomena and therefore controlling their sources is widely necessary for all lake management approaches (Ho and Michalak, 2017a; Lürling and Oosterhout, 2013; Su et al., 2016; Yenilmez and Aksoy, 2013). Their concentrations in the lake water and the ratio of N and P provide important data about the trophic state of the lake (Heisler et al., 2008; Wang and Wang, 2009). There are several examples worldwide of increases in harmful algal blooms linked to increased phosphorus loading (Heisler et al., 2008; Ho and Michalak, 2017b; Li et al., 2014). In fact, total phosphorus concentration, mainly the excessive bioavailability of $PO_4^{3-}$ form, has negative implications in algae and vascular plants growth in the overall water quality and biodiversity of the lakes (Ellison and Brett, 2006; Hein, 2006; Paerl and Otten, 2013). For that reason, P control is more feasible than that of N, since there is no atmospheric source of P available, unlike the case of N (Zamparas and Zacharias, 2014). However, source control of phosphorus forms is not always possible, especially if several diffuse sources are playing simultaneously. Although there are some evidences of lake responses to the reductions in external nutrient loading (Eigemann et al., 2016; Jeppesen et al., 2005; Tang et al., 2016), the internal P loading promoted by the continuous P release from bottom sediments apparently delays the recovery (Ahlgren et al., 2011; Dittrich et al., 2011; Lai and Lam, 2008; Lürling and Oosterhout, 2013). In fact, when the internal loading is so significant within a certain lake system, it can prevents the improvements in water quality and the water from the lake does not meet the established water quality criteria defined by the legal guidelines (Sondergaard et al., 2001). However, even after reducing the external loading, several techniques have been proposed to reduce the high internal nutrient loading aiming to recovery the limnetic ecosystem (please see section 1.4).

Another important factor that can directly or indirectly alter phosphorus availability and consequently threat shallow lakes dynamics is the impact of climate change. Although the lack of rigorous studies providing causal links between climate variability and phosphorus loading, there are evidences that both parameters are capable of influencing the eutrophication process and consequently the trophic lake status (Bertahas et al., 2006; Pérez-Ruzafa et al., 2005). Previous researches suggested that an

increase in surface water temperatures due to changing global climate are expected to interact strongly with existing increased phosphorus levels, where it can exacerbate a variety of eutrophication problems, such as the proliferation and/or expansion of harmful cyanobacteria blooms (O'Neil et al., 2012; Paerl and Huisman, 2008). More recently, the global warming was considered as a major trigger for the increase in geographic expansion and persistence of cyanobacterial species in temperate freshwater lakes (Scholz et al., 2017b). As a consequence of the massive occurrence of these blooms, a large decline of lake biodiversity and the dominance of few cyanobacterial species may occur (Bellard et al., 2012; Molot et al., 2014; Mooij et al., 2005; Sulis et al., 2014), having a serious impact on biological richness and diversity, as we previously described. Another important factor that could also influence the trophic state of lakes are the local hydrologic conditions, as simultaneous episodes of water inflow and outflow from/to lakes can be responsible for changes on phosphorus concentrations, affecting the biological communities (Bertahas et al., 2006). On the other hand, the occurrence of contrasting climatic fluctuations, such as the shifting between high rainfall precipitation and prolonged dry periods, have strongly influenced the water quality of the lakes (Parr and Mason, 2003; Touchette et al., 2007). In accordance, after drought periods the authors observed the reduction of lake's water renewal time and also the increase of nutrient loadings in the aquatic systems (Bertahas et al., 2006; Touchette et al., 2007).

In parallel with eutrophication and despite the seasonal dry periods, the rising sea levels and the increasing use of freshwater for agricultural irrigation are being responsible for increase the amount of salt entering aquatic freshwater ecosystems (Baldwin and Mendelssohn, 1998; Paerl and Otten, 2013). The salinity levels of a specific water body are dependent on water regime, in which the periods of increased salinity occur during low flow phases, while rainfall results in dilution of wetland salts or a flushing of salts from wetlands and floodplains to downstream channels (Brock et al., 2005; Nielsen et al., 2003). Also, under percolation processes, the salts could pass from the surface compartments to deep groundwater, thus helping the increase of salinity in the low deeper water layer (Li et al., 2016). In consequence of salinity and water regime interactions, the response of freshwater communities to this environmental pressure has

been deeply studied in limnetic systems. Investigations in wetlands from coastal marshes have pointed out that increased salinity (from 1 g $L^{-1}$ to 5 g $L^{-1}$) resulted in increased stress for the aquatic plants *Myriophyllum variifolium*, *Eleocharis acuta* and *Lythrum hyssopifolia,* with a decrease in species diversity and abundance (Baldwin and Mendelssohn, 1998; Nielsen et al., 2003). However, under eutrophic regimes, several common cyanobacterial bloom are salt-tolerant, as the case of *Anabaena*, *Anabaenopsis*, *Nodularia* and *Microcystis* (Bouvy et al., 2000; Mazur-Marzec et al., 2005; Tonk et al., 2007), but their presence may be governed by a combination of nutrient over-enrichment, climate changes and salinization as well (Paerl and Otten, 2013). Moreover, the dominance of these salt-tolerant species could occur as a response of high salinization levels observed in eutrophic systems. On its turn, comparisons of field data and laboratorial experiments indicated that salinity and cyanobacterial blooms influenced negatively the zooplankton population dynamics (Ferrão-Filho et al., 2002; Grzebyk and Berland, 1995). High salinity also reduce significantly the biomass of the aquatic macrophyte *Sagittaria lancifolia* (Baldwin and Mendelssohn, 1998) and imposed severe constrains to the growth and distribution of the *Typha domingensis* (Glenn et al., 1995). Nevertheless, when exposed to an environmental stress, salt-tolerant species with vegetative reproduction, as the case of plant *Spartina patens*, prevailed after the environmental disturbance, since they are more influenced by the water level rather than by the salinity (Baldwin and Mendelssohn, 1998). Thus, the recognised loss of wetland biodiversity is not only related with nutrient inputs, climate changes or salinization, but it is also a result of a complex combination of these parameters together with the hydrological conditions of the area.

Finally, the hydrogeological nature of the area can facilitate preferential groundwater flows and high spring discharges under the lake surface (Bertahas et al., 2006), whereas the local geomorphological and sedimentological data may provide the essential understandings about the long-term environmental change in wetlands induced by human impacts (Kotze et al., 2012; Pérez-Ruzafa et al., 2005; Tooth et al., 2009).

Portugal presents, according to Kopper classification (see https://www.ipma.pt/pt/educativa/tempo.clima/index.jsp?page=clima.pt.xml), a

Mediterranean temperate climate, in which winters are mild and summers are dry and moderately hot. Thus, in the Portuguese context, there are several evidences reflecting the eutrophication phenomena at particular habitats, which have been well documented by previous researches. The conditions of these systems are expected to be seriously influenced by the forecasted climate changes, especially for the Mediterranean regions, thus deserving a great attention. Some of the most demonstrative examples will be now described in (Figure I.1), based on useful indicators.


**Figure I.1.** Schematic representation and location of *Mondego* Estuary, Lake *Vela* and *Ria de Aveiro, Óbidos* and *Ria Formosa* lagoons

## 1.2. Portuguese Eutrophic Systems – the more relevant cases

### 1.2.1. *Mondego* estuary

Over the last decades, many Portuguese coastal water bodies have experienced a high anthropogenic loading of nutrients, responsible for a process currently named as cultural eutrophication (Ekdahl et al., 2004; Schindler, 2012; Smith et al., 2006). The major and one of the best documented examples is the *Mondego* estuary, a typical warm-temperate intertidal system located on the western coast of Portugal (Figure I.1). It is a mesotidal and well-mixed estuary, with a very irregular hydrological regime, with a low water flow during the dry period and strong freshwater discharges during the rainy season.

The *Mondego* estuary is about 7 km long and 2-3 km across at its widest part, presentes a cover area of 3.4 $km^2$ and is under a Mediterranean temperate climate. It is divided in two arms, north and south, separated by the *Murraceira* Island, which was formed by the deposition of detrital material transported by the river. The north arm is deeper (5-10 m during high tide, tidal range about 2-3 m), constitutes the main navigation channel, is the location of the *Figueira da Foz* harbour, and is mainly influenced by freshwater discharges of *Mondego* River. The south arm is shallower (2-4 m during high tide, tidal range 1-3 m) and is almost silted up in the upstream areas, which causes the freshwater of the river to flow mainly via the North arm. Consequently, the water circulation in the South arm is dependent on tides and on the freshwater input from the *Pranto* River, a small tributary. The discharge from this tributary is controlled by a sluice and is regulated according to the water needs of rice fields in the *Mondego* Valley. This freshwater input represented an important source of nutrients into the southern arm. Most of the intertidal area (75%) of South branch corresponds to mudflats, which are exposed during low tide.

*Mondego* estuary receives nutrient and chemical's rich-discharges from agricultural areas of upstream cultivated land in its drainage basin, mainly dominated by rice fields (Flindt et al., 1997; Lillebø et al., 2007; Marques, 1989; Martinho et al., 2007). In addition, this estuary supports discharges from several industries, many salt-works and aquaculture farms (Cardoso et al., 2004; Lillebø et al., 2007). The strong anthropogenic influence in the hydrographic system, such as the constructions of several infrastructures

to improve the main navigation channels, enlarge harbor facilities, and also the construction of small water reservoirs for agriculture and aquaculture purposes are some examples of the structural changes occurred in the last decades of 20$^{th}$ century (Dias et al., 1999; Flindt et al., 1997; Lopes et al., 2005; Marques et al., 2003; Martinho et al., 2007; Martins et al., 2001). As consequence, eutrophication become a problem and large macroalgal blooms, especially *Enteromorpha* or *Ulva* were recorded repeatedly, mainly at the South arm of the *Mondego* estuary (Dolbeth et al., 2003; Flindt et al., 1997; Lillebø et al., 1999; Marques et al., 2003; Martins et al., 2001, 1997; Patrício et al., 2004; Patrício and Marques, 2006). This was probably a result of excessive release of phosphorus and nitrogen into the estuary, coupled with their longer persistence in the water column (Flindt et al., 1997; Marques et al., 1997; Pardal, 1998; Pardal et al., 2000). Previous studies focusing on phosphorus dynamics and bioavailability at the *Mondego* estuary have shown that after reducing external point sources, phosphorus availability might persist in this particular eutrophic system as a result of sediment supply (Coelho et al., 2004). The low precipitation in the estuary along the years, reductions in turnover rates and increases in water stability, increases in temperature, salinity and light penetration also promoted the occurrence of green macroalgae blooms (Martins et al., 2001), which gradually replaced the resident rooted macrophytes (*Zostera* sp.) (Marques et al., 2003, 1997; Martins et al., 2001; Patrício et al., 2004). In accordance with data from water and sediments from the south branch of *Mondego* estuary, the trophic classification proposed by Wasmund et al. (2001) considered this system as eutrophic or polytrophic attending to the concentration of chlorophyll *a*, DIN and/or phosphate concentrations. Because the effects of different stressors influence biological integrity, particularly those related with eutrophication, the study of biological communities and consequently of the biodiversity have become a priority in the effective monitoring and assessment strategy of this system. In the *Mondego* estuary, the ecological quality has been assessed by examining the benthic fauna community due to its essential role in biochemical processes (Lillebø et al., 1999), and also as a bioindicator of the ecosystem health (Carvalho et al., 2006; Lillebø et al., 2007; Posey et al., 1995). According to the previous studies, the benthic macroalgae bloom of *Enteromorpha* influenced negatively the productivity of the isopoda *Cyathura*

*cariata*, a possible keystone species in the benthic community of the *Mondego* estuary (Martins et al., 1997). After the occurrence of seasonal *Enteromorpha* blooms, *C. cariata* biomass decreased dramatically probably by the strong anoxic conditions generated from the high algal decomposition rates. Also, Pardal et al. (2000) showed that such blooms had a strong negative impact on the life-cycle, population dynamics and production of the grazer amphipod *Amphitoe valida*, which could imply its disappearance from the *Mondego* estuary ecosystem. Thus, considering the results from research carried out in the south branch of the *Mondego* estuary (Cabral et al., 1999; Lillebø et al., 1999; Lopes et al., 2000; Marques et al., 2003; Pardal et al., 2000), it is reasonable to assume that eutrophication appeared as the major driving force behind the gradual changes in species composition at other trophic levels, giving rise progressively to a new trophic structure.

Based on field, experimental and historical information available, environmental models have been constructed to predict the eutrophication gradient along the southern arm of the *Mondego* estuary. Patrício and Marques (2006) used the Ecopath models to integrate available data about biomass, consumption and production, dynamics, food web and trophic flows along the estuarine network. Although these models could be further enhanced and revised, they represent the closest approximation about the eutrophication gradient along this particular system, considering the available data.

### 1.2.2. *Ria de Aveiro* lagoon

One of the Portuguese systems with a historic anthropogenic and industrial contamination is *Ria de Aveiro* coastal lagoon (Figure I.1). This system is located in the northwest Atlantic coast and is considered the most extensive shallow coastal lagoon in Portugal, with 45 km long and 10 km wide (Teixeira, 1994). It is a Special Protected Area, including several areas classified as Sites of Community Importance, is also considered as a special protection zone for birds, and hence was integrated in the Natura 2000 Network (EU Habitats Directive). The system is characterized by a series of channels between which lie significant intertidal areas, composed by mudflats, salt marsh and old saltpans that connect to the (Atlantic) ocean by an artificial inlet channel built in 1808 (Araújo et al., 2008). *Aveiro* urban area is connected directly with the lagoon through channels that

join the lagoon's main body over several canal locks (Martins et al., 2010). The main channels of this lagoon are *São Jacinto* and *Espinheiro* at the north, and *Mira* and *Ílhavo* at the south. Although these channels are interconnected, each of them may be considered as presenting features of distinct estuaries, particularly at the hydrological level, but also due to the nature, distribution and granulometric composition of sediments (Dias et al., 1999). The sediments from northern channels are fine cohesive, while those from southern have a sandy composition giving rise to different habitats in the lagoon (Borrego et al., 1994; Lopes et al., 2001). The main forcing action of these subsystems is the semidiurnal tides, while the strong wind conditions, which is very significant in *Aveiro*, may induce particular circulation patterns mainly in shallow areas and wide channels (Dias, 2001).

Biologically, this lagoon is a natural habitat for several fish and invertebrate species, provides natural conditions for navigation, tourism and recreation activities. However, the considerable development in the urban area, also made the lagoon a place of discharge of domestic and industrial wastes, which have increased the anthropogenic pressure on the system, contributing for the degradation of its water quality (Barrosa, 1985; Dias, 2001; Lopes et al., 2005). Additionally, its drainage basin offers good conditions for agriculture and cattle activities, and this system is consequently rich in nutrients and organic matter (Lopes et al., 2005). In agreement to these authors, high levels of nitrogen ($NO_3$ and $NH_4$) and phosphorus compounds ($PO_4$), high temperature and light intensity are the factors responsible for the phytoplankton development and for the several environmental problems observed in this system, particularly the eutrophic conditions established in the last decades. A pattern of successive algal blooms was registered from late autumn 2000 to late summer 2001, and the effects of eutrophication on seasonal succession of phytoplankton may be dependent on nutrient concentrations and composition (Lopes et al., 2007). However, the permanent connection to the ocean allows a constant renewal of dissolved oxygen conditions within the lagoon and also influence nutrients distribution (Lopes et al., 2005). The strong mixing of the water masses is tidal forced, and the properties of tides and tidal currents have been deeply described in several mathematical models (Araújo et al., 2008; Dias et al., 2000, 1999;

Dias and Lopes, 2006; Lopes et al., 2001). The effects of their flows induce both the erosion of sediments by high current speeds in the shallow areas determining sediment transport in the lagoon with impacts in nutrients cycling (Lopes et al., 2001).

Previous research highlighted the metal contamination in the lagoon, in which high concentrations of Hg, Cd and Cu were observed in sediments and water from internal areas of *Ria de Aveiro*, particularly at northern channels, probably by the ability of the fine cohesive sediments to form complex and stable chemical structures with metals (Martins et al., 2010; Monterroso et al., 2007; Pereira et al., 1997). In consequence of metal contamination, producers present a substantial incorporation of mercury in their tissues due to Hg exposure of aquatic food webs, as it was demonstrated by Coelho et al. (2005). According to this research, the dominant species of macroalgae in *Ria de Aveiro* revealed a total mercury content ranging from 0.02 to 2.1 μg $g^{-1}$ dw. *Fucus* was the algae with the highest bioaccumulation potential, followed by *Gracilaria* and *Enteromorpha*. Also, considering the ongoing eutrophication process in the *Ria the Aveiro* coastal lagoon and the dominance of these macroalgae species in the ecosystem, the occurrence of blooms represent a substantial source of mercury as a result of its reported capacity to bind trace metals (Coelho et al., 2005; Radway et al., 2001; Vasconcelos and Leal, 2001). In consequence, producers including seagrass species (*Zostera noltii*) and salt marsh plants are efficient in accumulating, transporting and transferring mercury through the estuarine trophic webs (Coelho et al., 2009). Despite the water pathway, mercury can also be transferred to higher trophic levels through direct uptake from contaminated sediment by sediment-dwelling invertebrates (*Scrobicularia plana* and *Hediste diversicolor*), due to their omnivorous diet, burrowing activities and uptake in anoxic sediments (Coelho et al., 2008). Abreu et al. (2000) also reported a high accumulation of mercury in the tissues (muscle and liver levels up to1 μg $g^{-1}$ and 2 μg $g^{-1}$, respectively), and also in the gills and stomach (contents of 0.71 μg $g^{-1}$ and 0.43 μg $g^{-1}$ in gills and stomach, respectively) of *Dicentrarchus labrax* (sea bass) captured in *Ria de Aveiro* with a trend of increasing mercury accumulation with age. Although the recognized impact of metal contamination at different trophic levels, the presence of pesticides is also a threat for *Ria the Aveiro* ecosystem (Lopes et al., 2001). However, the most evident cause of

degradation of the system is linked with eutrophication, thus the control and management of nutrient inputs is the first step required for ecosystem rehabilitation, particularly for improving water quality.

### 1.2.3. *Ria Formosa* lagoons

The *Ria Formosa* is a shallow large coastal lagoon in the south coast of *Algarve* (Figure I.1). It has been recognised as an important natural wetland with great economic value, classified as a natural park since 1987 and accepted as a Ramsar and Natura 2000 site. Considering the Coordination Commission of the Algarve Region (CCRA, 1984), the total area of the *Ria Formosa* National Park is about 18,000 ha including a 10,000 ha coastal lagoon with salt marshes and mud flats (5000 ha), sand banks and dunes (2000 ha), saltpans and aquaculture ponds (1000 ha). The *Ria Formosa* includes a large intertidal zone of about 55 km (east-west) and 6 km (north-south). This lagoon is separated from the sea by two peninsular sand spits and also by a string of barrier islands, but is still connected to the sea by several inlets that allow exchanges of water with the Atlantic Ocean (Newton and Mudge, 2003). The mean air temperature in summer and winter in this region is 25ºC and 12ºC, respectively, and although *Ria Formosa* is located on the Atlantic coast, its climate is predominantly Mediterranean, with dry and hot summers, and warm and wet winters. In contrast to Mediterranean lagoons, *Ria Formosa* is mesotidal with a semi-diurnal tidal regimes, with tidal variations from 1.35 m on neap tides to 3 m on spring tides (Instituto Hidrográfico, 1986). Due to the complex network of navigable channels, the lagoon is a strategic resource for the region, particularly for tourism, fisheries, aquaculture, salt and sediment extraction activities (Newton and Mudge, 2005, 2003). However, the navigable channels of the *Ria Formosa* were extensively dredged in the last years with an average depth of about 6 m (Newton and Mudge, 2003), while the average water depth of the lagoon is significantly lower, being less than 3 m (Mudge et al., 1999).

This system has been the target of several pressures which deteriorated the water quality, as the documented contamination by metals and organochlorine compounds (Bebianno, 1995; Cortesão et al., 1986), as well as the increased nutrient loadings along

the years. Much of this has been related to the poorly threated domestic sewage discharged on the area, as well as to the non-point source agricultural runoffs that lead to eutrophication and subsequent decrease in dissolved oxygen levels (Mudge and Bebianno 1997; Newton and Mudge 2005; Newton et al. 2010). Existing data on nutrients in the water column revealed a significant enrichment in nitrogen ($NH_4^+$, $NO_2^-$ and $NO_3^-$) and phosphate ($PO_4^{3-}$) (Loureiro et al., 2006; Newton and Mudge, 2005) in inner areas of the lagoon, indicating that these areas present a low water renovation rate, particularly in spring and autumn (Newton and Mudge, 2003). Given the shallowness of the lagoon, sediment-water exchanges are also considered to be an important process in the nutrient dynamics in the lagoon (Brito et al., 2010; Murray et al., 2006; Serpa et al., 2007). According to these studies, the large concentrations of pore water nutrients (i.e. ammonium, nitrate, phosphate and silicate) have been likely released by sediments, and has been responsible for the "poor" or "bad" ecological quality status of the *Ria Formosa* (Newton et al. 2003). In fact, benthic eutrophication symptoms such as large microalgae biomass and strong dissolved oxygen fluctuations in the tide pools increased in the last years (Brito et al., 2010; Nobre et al., 2005; Padinha et al., 2000). In this context, using phytoplankton biomass as indicator, Cartaxana et al. (2009) classified two freshwater lagoons of the *Ria Formosa* (*Dunas Douradas* and *Garrão*) as "hypereutrophic" with dominance of diatoms and chlorophytes, and the *Salgados* brackish lagoon was also considered "hypereutrophic", but with a dominance of cyanophytes. Finally, *Almargem* lagoon was classified as "mesotrophic", being the diatoms the most representative group of phytoplankton community. The obtained classifications reflected the domestic discharges from nearby wastewater treatment plants, as well as the diffuse pollution (Cartaxana et al., 2009; Coelho et al., 2007). In fact, there are historical evidences of eutrophication symptoms in the *Ria Formosa*, mainly by the incidence of harmful algal blooms (Baptista, 1993). Along the last years, the variations of the fish community of the *Ria Formosa* was also used as biological indicator of human induced changes in the system (Ribeiro et al., 2008). These changes were mostly related to a decrease in the abundance of Mugilidae from the period of 1980-1986 to 2001-2002, probably by a decrease in the organic matter content and nutrient concentrations, as a result of the

improvements in sewage treatment and better water circulation inside the lagoon. Although capable of feeding on invertebrates and plankton, the Mugilidae are mainly detritivorous, filtering, ingesting, concentrating and assimilating large quantities of organic matter, including that of sewage origin (Laffaille et al., 2002). Also, Ribeiro et al. (2008) highlighted that changes in the fish community structure were more evident in the inner areas of the lagoon than near the inlet, because inner parts of the lagoon are poorly mixed, being also exposed to a high anthropogenic pressure. Furthermore, several indigenous species collected from the *Ria Formosa* lagoon are impacted by diverse contaminants, as the case of the polycyclic aromatic hydrocarbons (PAHs) and organotin compounds (TBT) detected in clams (*Ruditapes decussatus*) (Barreira et al., 2007; Coelho et al., 2002), PAHs and metals in mussels (*Mytilus galloprovincialis*) (Bebianno and Machado, 1997; Cravo et al., 2009), and also by a mixture of contaminants detected in shore crabs (*Carcinus maenas*) (Maria et al., 2009).

### 1.2.4. *Óbidos* lagoon

*Óbidos* lagoon is a eutrophic and metal-contaminated shallow coastal ecosystem located on the western Portuguese coast, with a mean depth of 1 m and a surface area of 7 $km^2$, permanently connected to the Atlantic sea through a narrow inlet (Carvalho et al., 2006) (Figure I.1). Environmental data of *Óbidos* lagoon, including temperature, salinity, dissolved oxygen and nutrients, as well as the ecological quality of water during open vs. closed regimes were reported by Coutinho et al. (2012), highlighting the evolution of the system from 1958 to 2010. The system comprises areas of different morphology and sedimentology, with sand banks and narrow channels in the lower lagoon; a mixture of sand and mud in the middle lagoon, and with muddy bottom sediments in the upper lagoon (including *Bom Sucesso* branch and *Barrosa* branch) (Pereira et al., 2009a). Similarly to the other Portuguese coastal lagoons, the water circulation within the *Óbidos* lagoon is mostly driven by tidal energy whose tidal amplitude ranged between 1 and 2m (Oliveira et al., 2006). From all the branches that compose the *Óbidos* lagoon, the *Barrosa* branch is the shallower (mean depth 0.5-1m) and it receives discharges from agricultural adjacent fields and urban effluents from a nearby town named *Caldas da Rainha* (Pereira

et al., 2009a). In consequence, this branch presents a high nutrient availability, being considered “eutrophic” to “polytrophic” (Pereira et al., 2009c), with abundant macroalgae (*Ulva* sp. and *Enteromorpha* sp.) and a wide daily variation of dissolved oxygen levels during summer (Pereira et al., 2010, 2009b). The dissolved oxygen levels in *Barrosa* branch tends to decrease to low saturation values indicating hypoxic conditions at summer (Pereira et al., 2010). More recently, winter-summer fluctuations on the nutrient composition reflected an excess of phosphate in the summer season, mainly attributed to phosphate regeneration from sediments of the upper lagoon with accentuated symptoms of eutrophication (Pereira et al., 2012). According to this research, high levels of dissolved organic nitrogen and dissolved organic phosphorus also appeared at higher concentrations in summer, possibly reflecting the increase of organic matter degradation under high temperature. Particularly in *Barrosa* lagoon, the increase of organic forms of N and P was the promoting factor to the phytoplankton production. Concentrations of chlorophyll *a*, dissolved inorganic nitrogen and phosphate are within the range of values reported for other estuaries in the same region that also receive nutrients from diffuse and local sources, as the case of *Mondego* estuary (Lillebø et al., 2005) and *Ria de Aveiro* (Lopes et al., 2007). Furthermore, the decomposition of the abundant *Ulva* sp. and *Enteromorpha* sp. senescent biomass is also contributing for the eutrophic status of this water mass (Carvalho et al., 2006; Pereira et al., 2012). These conditions in the lagoon have also favored the occurrence of blooms of toxic diatoms (mainly dominated by *Gymnodinium catenatum*, *Dinophysis acuminata* and *Dinophysis acuta*), which were responsible for the recurrent episodes of shellfish toxicity in the *Óbidos* coastal lagoon, as well as along the Portuguese coast in the summer and early autumn (Botelho et al., 2010; Vale et al., 2008). Despite the symptoms of eutrophication caused by the nutrients input, the concentration of metals in water and sediments are also high in *Barrosa* lagoon, whose source is a small tributary (*Cal* River) contaminated by agro-industrial activities. Once again, anoxic conditions recorded during the period of night, in the summer, favors the exportation not only of nutrients, but also of metals (Fe, Mn, $Si(OH)_4$, Cr and Co) from the sediment to the water (Pereira et al., 2010, 2009b, 2009c). Although the difficulty to establish cause-effect relationships due to the occurrence of various stressors and their

interactions, the high impact of eutrophication and moderate metals contamination registered in *Barrosa* water were associated with the oxidative stress responses (at CAT, GPx, GST, $GSH_t$, LPO and EROD levels) detected in the shore crab *Carcinus maenas* (Pereira et al., 2009a). These results suggest that the combined effects of eutrophication and metal contamination could have impacts at aquatic species from different trophic levels, likely affecting the biodiversity of the system.

### 1.2.5. Lake *Vela*

Lake *Vela* is a eutrophic shallow system located in the littoral-center of Portugal, about 6 km far away from the Atlantic Ocean, with a mean depth of 0.9 m and 70 ha of floodable surface area (Figure I.1). This lake is integrated in a larger system of interconnected reservoirs, usually designated by the "*Quiaios* lakes", parallel to the coastline, in a sand-dune area (Abrantes et al., 2006b). This freshwater resource was included in a site (PTCON0055) of the Natura 2000 Network, according to the resolution of the Council of Ministers 76/2000. This lake was also classified by the Municipality of *Figueira da Foz* as a first level natural area requiring protection measures (Abrantes et al., 2006b).

A pine tree forest (dominated by *Pinus pinaster* and *Acacia* spp.) surrounds the Lake *Vela* in the west side, while agricultural fields and some human settlements surround the east side (Fernandes, 1999). According to Koppen classification (see https://www.ipma.pt/pt/educativa/tempo.clima/index.jsp?page=clima.pt.xml), the lake is included in a region with a temperate Mediterranean climate with mild winters, in which the annual precipitation is of 801-1000 mm and the annual mean temperature is about 15.1-16ºC. Also, summers are characterized as dry and moderately hot, and a long wet period occurred between October and February (Abrantes et al. 2006b). Due to the sandy nature of the soils (with low organic matter content and high porosity), the fields are abundantly fertilised with livestock manures, chemical fertilizers and agrochemicals, according to inquiries realised to local inhabitants by Pereira (1997). Furthermore, the topography of the system, the low depth of the aquifer and the existence of water channels draining to the lake (Fernandes, 1999), are site-specific characteristics that enhance the risks of diffuse contamination in Lake *Vela*, mainly through leaching and

runoff from adjacent agricultural fields (Abrantes et al., 2008, 2006b). As a consequence of the regular inputs of nitrogen and phosphorus forms with direct implications on phytoplankton biomass, this lake is considered highly "eutrophic". Furthermore, despite eutrophication, this system has also been threatened by repeated introductions of alien fish species (i.e. *Lepomis gibbosus*, *Gambusia holbrooki* and *Cyprinus carpio*) that potentially change fish-zooplankton interactions. The study performed by Castro et al., (2007) suggested that the lower abundance of herbivorous zooplankton (i.e. *Daphnia*) in Lake *Vela* was a typical indicator of the strong fish predation pressure, fact that probably compromise the predation forces by zooplankton in regulating the abundance, biomass and species composition of algae, i.e. in the top-down control of the phytoplanktonic community (Jürgens et al., 1999). In the last decades, algal blooms have become increasingly frequent in Lake *Vela* (Abrantes et al., 2009, 2006a, 2006b; Antunes et al., 2003; Castro and Gonçalves, 2007; de Figueiredo et al., 2006). These blooms were especially dominated by several strains of the harmful cyanobacteria *Aphanizomenon flos-aqua* and *Microcystis aeruginosa* that have the ability to produce toxins, endangering a wide variety of organisms (Barros, 1994; de Figueiredo et al., 2006; Fernandes, 1999; Vasconcelos et al., 1993). Additionally, the bloom's collapse with a rapid oxygen depletion, leading to hypoxia or even anoxic conditions, have been potentially responsible for the large fish kills previously documented in the system (Abrantes et al., 2006a; Castro and Gonçalves, 2007; de Figueiredo et al., 2006; Pereira, 1997). As a consequence, a decline on aquatic biodiversity has been observed in this lake, not only caused by the eutrophication processes, but also by the predatory pressure of the introduced alien piscivorous fish species under the native ones. Because of this fact, the indigenous cyprinid community (*Barbus* spp., *Chondrostoma* spp. and *Squalius* spp.) were displaced by such an exotic assemblages and the Lake *Vela* fish community had changed radically (Castro et al., 2007).

Finally, as part of an ecological risk assessment of the Lake *Vela* area, evidences were collected of diffuse contamination by active ingredients of pesticides, namely aldrien, dieldrin, alachlor and glyphosate, in surface water and sediments from the east

side of the lake (Abrantes et al., 2010, 2009, 2006b), and also in groundwater (Abrantes et al., 2010).

### 1.3. European water quality regulations and its applicability in some of the Portuguese eutrophic systems

The growing concern on the quality of water resources and their eutrophication related problems lead the development of some remarkable European environmental policies. One of the most important legal instruments in force presently is the European Water Framework Directive 2000/60/EC (EU-WFD), whose main goal is to achieve a good ecological status in all European water bodies during the second and third management cycles of 2015-2021 and 2021-2027, respectively (European Commission, 2012). The purpose of this Directive is to establish a framework for the protection of inland surface waters, transitional waters, coastal waters and groundwater. Focusing on the case of coastal waters, this Directive involves lagoons with different gradient of salinity and also the shallow freshwater lagoons.

In Portugal, to address the implementation of the EU-WDF, all lagoons were considered coastal waters and were divided in two different typologies according to their permanent or temporarily communication with the sea (Brito et al., 2012b; Coutinho et al., 2012). Generically, and according to this classification, the open lagoons are connected to the sea throughout the year and are located in the southern Portugal coast (the most relevant example is *Ria Formosa*); the semi-closed lagoons, which are temporarily separated from the ocean by sand barriers and are located in the western coast of central Portugal. This division is consistent with the classification presented by Tagliapietra and Ghirardini (2006), which related the southern lagoons with most of the Mediterranean ones (hot summer Mediterranean climate with dry season and precipitation during the winter) and the systems located to the north of Lisbon with the ones in the Galician coast (Mediterranean climate with dry season during warm summer).

Due to the high natural dynamic of the coastal lagoons, the analysis of their ecological quality is a challenging process. For that reason, the EU-WDF required the definition of reference conditions for each biological element in order to assess the

ecological status of surface water bodies. In the case of lakes, there are several indicators that can be used in assessing their ecological quality, such as the composition and abundance of phytoplankton, macrophytes, phytobenthos and macroinvertebrates. The composition, abundance and age structure of the fish community should also be taken in consideration (Moss, 2007). This author also highlighted the water chemistry (nutrients and pH), morphology, hydrology, temperature, salinity, oxygen status and the detection of pollutants, as other indicators that should be included in order to perform a correct evaluation. Thus, phytoplankton is generically one of the most important biological indicators for the assessment of the ecological quality of superficial water bodies, since one of the first symptoms of eutrophication is an enhanced algal biomass as part of phytoplankton blooms. These blooms are evaluated having in consideration the taxa composition, the intensity and frequency. Also, nutrient loads, mainly nitrogen and phosphorus, is of a greater significance since their effluxes from sediments to the water column might contribute for the enrichment of waters and for the intensification of the eutrophication process (Brito et al., 2010, 2012b; Pereira et al., 2009c). These biological standards helped to identify the ecological status of a certain water body, considering the 5-class scale boundaries (high, good, moderate, poor and bad) previously established by the EU-WFD.

Several Portuguese coastal lagoons were assessed under the Water Framework Directive requirements, particularly at the level of phytoplankton biomass (chlorophyll *a* concentration) and the frequency of phytoplankton blooms, both at open and closed regimes. In the *Óbidos* lagoon, there were significant differences related with chlorophyll *a*, whose concentrations varied from 0.7 µg $L^{-1}$ to 63.7 µg $L^{-1}$ when the lagoon was connected to the sea (i.e. open), and from 0.2 µg $L^{-1}$ to 289.3 µg $L^{-1}$ in the closed periods (Coutinho et al., 2012). These authors reported that a reference value for phytoplankton biomass during the open periods was set to be 6.7 µg $L^{-1}$. Furthermore, the inner and shallow branch of the *Óbidos* lagoon (*Barrosa*) was the most productive area in terms of phytoplankton abundances and nutrient increase, as well as in the frequency of harmful algal blooms occurrence under closure periods (Coutinho et al., 2012). After applying the WFD classification system regarding the phytoplankton and nutrient indices, the part in

contact with coastal waters was classified as in "high" quality status, while the inner part of the lagoon (the *Barrosa* branch) was in "good" status, only for the open regime (Coutinho et al., 2012; Pereira et al., 2009c). Considering the indexes proposed by Crouzet et al. (1999) for the assessment of trophic classification, the *Barrosa* branch was classified as "bad" based on phosphate, nitrate + nitrite, while the inlet and middle areas of the lagoon registered a better quality status being classified between "poor" to "fair" (Pereira et al., 2009c).

Following a long eutrophication process that started by the early 1980s, experimental mitigation measures were implemented in 1998 on the *Mondego* estuary. The approach followed consisted in reducing the opening of the sluice connecting a small tributary to minimise the nutrient loading within the estuary, together with the improvement of system hydrodynamics by engineering work in the upstream areas (Lillebø et al., 2005). The results obtained by these authors confirmed the effective reduction in the dissolved N:P ratio from 37.7 (in the period from January 1993 to January 1997) to 13.2 after 1998 (in the period from January 1999 to January 2003), which promoted a significant reduction of green macroalgae biomass, followed by an increase of seagrass *Zostera noltii* biomass and covered area. No relevant changes in the oxidised forms of nitrogen (nitrate + nitrite) or in the concentrations of the dissolved inorganic phosphorus between pre- and post-mitigation periods were observed. More recently, the effect of the mitigation practices implemented in 1998 was evaluated, based on several classifications methods for water quality status, attending to the monitoring requirements available for the south arm of the *Mondego* estuary. The physico-chemical status of the water quality of this particular area, according to the EU-WFD, was considered "moderate" during 1993-1994, whilst improved to "good" in 2000-2001, attending to the annual means of nitrogen, phosphorus and percentage of oxygen saturation (Lillebø et al. 2007). In addition, the same study revealed that the system improved significantly on its overall eutrophic condition after mitigation measures have been implemented. Although the mentioned parameters are important indicators in assessing the ecological status, the study of benthic communities within EU-WFD scope would also be useful in the *Mondego* estuary analysis, since these have been well-known

issues of opportunistic macroalgae blooms (Ferreira et al., 2006; Teixeira et al., 2007). In fact, several ecological indices (AMBI, Margalef and Shannon-Wiener) were applied to the benthic communities and different ecological situations were detected in different areas of the *Mondego* estuary (Teixeira et al., 2008, 2007). Additionally, these studies also highlighted the need in establishing benthic reference conditions values through the estuarine system. More recently, during a sampling period in 2008, different water bodies from the *Mondego* estuary were classified as having a "high" to "poor" ecological status, only based on the phytoplankton biomass (as chlorophyll *a*), due to the lack of information of phytoplankton blooms (Brito et al., 2012a).

Although *Ria de Aveiro* was not listed as a sensitive area (Directive 91/271/EEC) or vulnerable zone (Directive 91/676/EEC) in the report performed by Portuguese Water Institute (INAG) two decades ago, the same report concluded that *Ria de Aveiro* had a "moderate" degree of eutrophication, a "high" level of chlorophyll *a* and a "high" load of nutrients into the system. These could be result of the use of septic tanks or other small scale systems for sewage disposal, with poor treatment, within the *Ria de Aveiro* watershed (Lopes et al., 2017). In order to reverse this situation, significant investments were made in the lagoon by the company responsible for the treatment of sewage and industrial effluents in the *Ria de Aveiro* (SIMRIA – Saneamento Integrado de Municípios SA). In 2005, the company implemented a new regional multi-municipality sanitary system with secondary wastewater treatment, which was connected to a submarine outfall (S. Jacinto outfall), in order to reduce the sewage drained directly to the lagoon. This measure occurred during the first WFD management cycle (operated from 2009 and 2015) and six sites distributed all over the lagoon were evaluated, aiming to analyse the impact of this measure in the lagoon, from 2005. Published data on dissolved inorganic nutrients, phosphate and chlorophyll *a* collected in 2001 (Lopes et al., 2007) were compared with data collected in 2012 (Lopes et al., 2017). Showed an overall decrease in concentrations of dissolved inorganic nitrogen (DIN), particularly the contribution of ammonium form of nitrogen ($NH_4$-N), since the ammonium reduced from 30% in 2001 to 3% in 2012. This reduction included both non-point sources, such as the runoff from agricultural areas, and point sources like effluents and emissions from several industries

and livestock's farms (Lillebø et al., 2005; Lopes et al., 2007). Moreover, a generic decrease in winter phosphate ($PO_4$-P) occurred, probably by the reduction in exogenous point sources of this nutrient (Lillebø et al., 2005, 2004). However, the increases of phosphate in summer 2012 in the lagoon inner areas was likely the result of fluxes between sediment and water column, which could be promoted by a combination of environmental factors, as higher water temperatures (maximum 22ºC in 2001 and 24.6ºC in 2012) and lower hydrodynamics (Otero et al., 2013). Chlorophyll *a* also showed clear seasonal, spatial and inter-annual variations, with higher mean concentrations in 2001 (ranging from 1.1 $\mu gL^{-1}$ to 12.6 $\mu gL^{-1}$ in 2001 and from 0.6 $\mu gL^{-1}$ to 6.2 $\mu gL^{-1}$ in 2012). The introduction of the mitigation measures as the case of the new multi-municipality sanitary system, with secondary treatment connected to the *São Jacinto* outfall, had effectively reduced the point source nutrient loads, which contributed to the eutrophication abatement (Lopes et al., 2017). In fact, considering the phytoplankton biomass (as chlorophyll *a*), Brito et al. (2012a) reported that several water bodies from *Ria de Aveiro*, particularly those channels that connected to the Atlantic ocean, were classified as having a "high" ecological status, but the upper part of the *Ria* (in the *Ovar* channel) was classified as being in a "poor" ecological state, probably by the high waters residence times and accumulation of nutrients (Leandro et al., 2007; Lopes et al., 2007). Although the overall ecological condition of the *Ria de Aveiro* was considered "moderate" (Brito et al., 2012a), the lagoon remains exposed to non-point nitrogen sources as a consequence of land use and water management, and this fact are not expected to change in this natural system (Lopes et al., 2017). Regarding to the quality of the sediments of this system, a recent ecotoxicological analysis was performed, and although sediments were not considered at risk based on National Sediment Quality Criteria (Portaria nº1450/2007 de 12 de Novembro de 2007, Diário da República), bioassays performed with sediment samples or sediment elutriates revealed to be highly toxic for marine species (*V. fischeri* and *B. plicatilis*) (Gonçalves et al., 2013). According to this research, an ecotoxicological approach should be considered to complement more conservatives approaches and highlights currents limitations within the WFD assessment tools.

Water nutrients and phytoplankton quality elements were used to compare the conditions of an oceanic inlet and two stations (*Ramalhete* at the inner part and *Ponte* that is located on a channel that links the oceanic inlet with the blind-end of the western lagoon), within the *Ria Formosa* lagoon, according to the EU-WFD. Loureiro et al. (2006) observed a high nutrient enrichment in both areas, but a highest accumulation of biomass in the inner regions (i.e. *Ramalhete*) where water circulation is restricted, leading to episodes of water quality degradation. Thus, on the basis of historical data related with the mentioned parameters, the sites *Ramalhete* and *Ponte* have been classified with an ecological status of "good/moderate" and "high/good", respectively (Goela et al., 2009; Loureiro et al., 2006). The analysis of water samples collected from 2006 to 2008 showed a decrease in nitrogen and nitrate + nitrite concentrations in the water column (Brito et al., 2010), which may represent an improvement in water quality, particularly compared with results from Newton et al. (2003) in samples collected from June 1987 to May 1888 inclusive. Following the criteria provided by the Commission Decision 2008/915/EC (European Communities, 2008), *Ria Formosa* system had a "high" ecological quality in 2006-2007 (Brito et al., 2010), as well as in 2009-2010 (Brito et al., 2012b), considering all available phytoplankton data (chlorophyll *a*: selected species composition and abundance). The quality status based on phosphate levels was most of time considered "good" or "moderate" in 2007-2008, fact that is still considered to be lower than the target objective (i.e. "good" status) defined by the Water Framework Directive for 2015 (Brito et al., 2010). In fact, in order to understand nutrient dynamics and to evaluate the lagoon vulnerability to eutrophication, Brito et al. (2010) demonstrated that sediment-water exchanges are the most important processes in nutrient dynamics of the lagoon, particularly in regions of restricted water exchange. Thus, since sediments act as the main sink of nutrients within the lagoon (Brito et al., 2010; Falcao and Vale, 1998; Murray et al., 2006), the standard monitoring plans required by the Water Framework Directive should be carefully assessed, as they may fail to track relevant changes in the nutrient conditions and dynamics, as well the microalgae responses to them.

In the Lake *Vela*, despite it is part of Natura 2000 Site PTCON0055 according to the Resolution of the Council of Ministers 76/2000, there are no data available related to the

evaluation of the ecological quality status of water, as well as with the application of measures to improve the ecological condition of this freshwater system, which has becoming seriously compromised in the last decades.

### 1.4. Generic physicochemical mitigation methods used to reduce eutrophication symptoms worldwide

Several remediation strategies have been implemented worldwide to face the eutrophication problem, mostly by the P scavenging or removal from bottom sediments. These strategies include physical and chemical approaches, and their application can occur alone or combined, considering the needs and the rehabilitation scenario defined for any particular eutrophic freshwater.

In smaller lakes and reservoirs, the aeration/oxygenation is the physical method mostly applied in the recovery and should be started when oxygen concentrations fall below 6 $g/m^3$ in the hypolimnion in spring (Hickey and Gibbs, 2009). This method regulates P and N in the water column through the oxygenation of water, limiting the dominance of cyanobacteria and surface scum formation (Toffolon et al., 2013). However, the hypolimnetic aeration is applied in shallow lakes (without mixing), while the whole lake aeration (full mixing) is recommended for deep lakes (> 10 m) (Engstrom, 2005; Grochowska et al., 2013). Analysis of several results showed that artificial aeration has caused a radical decrease of nutrients and organic compounds content in the water (mainly due to halted release of phosphate from the bottom sediments), and eventually improved the trophic state of eutrophic shallow lakes (Bryant et al., 2011; Grochowska and Gawrońska, 2004; Horppila et al., 2015; Liboriussen et al., 2009; Toffolon et al., 2013). Although the main advantage of aeration is the rapid reoxygenation of the lake, the interruption of this method may rapidly revert the previous conditions of the lake, fact that represents the main disadvantage of the method (Hickey and Gibbs, 2009). In this context, recent researches observed that experimental cessation of hypolimnetic oxygenation drastically changed the lake's temperature regime and dissolved oxygen regimes, but it did not significantly affect nutrient conditions and its trophic status (Kuha et al., 2016; Salmi et al., 2014). In order to improve the longevity of the treatment

effectiveness, aeration can be complemented with other remediation approaches, such as the application of sediment capping agents (Hickey and Gibbs, 2009; Ross et al., 2008; Spears et al., 2013).

The sediment dredging represents another physical technique applied to small lakes restoration and consists in removing surface sediment layers using engineering structures. This approach also targets both P and N through the permanent removal of small areas of highly enriched sediments and also contaminated sediments. Several studies have documented the dreading efficacy in short-term, over the motorization of pre-and post-dredging conditions, since a general decrease of internal pollutant source occurred in eutrophic shallow lakes (Reddy et al., 2007; Yenilmez and Aksoy, 2013; Yu et al., 2017). The sediment dredging involves high costs by the use of large and specialized machinery, but the dredging technique directly *per se* also promotes the release of the dissolved organic matter that originally is embedded in deeper sediment layer into sediment-water interface and the overlaying water column, fact that may compromise the lake integrity (Jing et al., 2013; Xu et al., 2016; Zhang et al., 2010; Zhong et al., 2010). For this reason, the dredging application is controversial, but its effectiveness may be possible for non-geothermally influenced lakes, where the sediment becomes a source of fertiliser for agriculture (Hickey and Gibbs, 2009).

With respect to the control of the internal loading based on chemical remediation treatments, capping agents are used to permanently block the release of P and N from the sediments below the capping layer. According to the nature of the materials, capping agents are considered as passive or active. The sand, gravel and clay are examples of passive barriers and their ticker and finer layer enhance burial of organic matter, thus reducing sediment oxygen and the efflux of nutrients (both P and N) from the sediment (Cooke et al., 2005). Moreover, these capping materials improve the benthic habitat (in particular for the sediment-dwelling species) and allow the recolonization of benthic community, and for these reasons it represents an efficient barrier between the contaminated sediment and the environment where the level of oxygenation is high enough for the benthic settlement (Bona et al., 2000). On the other hand, active capping agents are chemical or geochemical materials capable of binding contaminants by

adsorption or precipitation processes, mainly by the reduction either of $NH_4$-N and DRP (i.e. dissolved reactive phosphorus) from sediment (Cooke et al., 2005; Hickey and Gibbs, 2009). These active barriers include natural products, such as allophane and aluminium sulphate (alum), and modified natural products as the case of Phoslock$^{TM}$, lanthanum (La) and zeolites.

Although alum or aluminium sulphate ($Al_2(SO_4)_3$) is known to be used as a sediment capping material, its primary function is as flocculation agent (Cooke et al., 2005). In Europe and United States, alum is the most common flocculation agent in the water treatment and it has been used in water clarification and lake restoration for a long time. Towards the end of the summer, when the bottom lake water is anoxic and the accumulation of dissolved reactive P is maximum, the alum successfully binds with freely P content (P precipitation) available in the water, ensuring the control of sediment P release (P inactivation) (Cooke et al., 1993). Depending on pH (alum optimal pH performance is above 6 and up to 8) (Zamparas and Zacharias, 2014), phosphorus precipitation removes phosphates from the water column, but during its settlement some algae and other suspended solids can also be trapped resulting in clearer water (Jeppesen et al., 2005). Used at the correct dose and considering the lake morphology (e.g. depth, stratified or shallow lakes, watershed lake area), the capping layer formed on the sediment is expected to remain effective for at least 5 up to 20 years in some lakes (Hickey and Gibbs, 2009; Huser et al., 2016), showing the long term success of alum treatment. In accordance, research over the past decades has shown that alum field applications have generally led to long term improvements to lake quality, considering the significant reduction of internal P release from deeper sediments in several eutrophic lakes (Cooke et al., 2005; Huser et al., 2011; Paul et al., 2008; Smeltzer et al., 1999). However, Han et al. (2013, 2012) reported that alum treatment caused *Microcystis* cell damage and subsequent release of large amounts of microcystin-LR, and also a decrease in the activity of other cyanobacteria-related bacteria, suggesting that alum treatment could be not suitable for removing toxic cyanobacterial blooms in lakes. In contrast, the use of allophane, particularly as a nanoparticle, has a large capacity for absorbing and

precipitating phosphates in aqueous solutions, and Yuan and Wu. (2007) considered this natural nanoclay as highly promising in the remediation of eutrophic waters.

Concerning lake recovery using modified natural products, Phoslock™ is a lanthanum-modified bentonite clay, and it works as a sink for phosphorus in water by forming lanthanum (La) phosphate, a compound with low solubility (Yuan and Wu, 2007). Owing to Phoslock™ flocculent capacity and their rapid dispersion in lake water, this product removes the dissolved reactive phosphorus (DRP) from the water and blocks the efflux of DRP from lake bed sediment to the overlaying water (Hickey and Gibbs, 2009). P removal by Phoslock™ is optimum when the lake is fully mixed and at pH values between 6 to 10, although adsorption occurs between 4.5 and 8 with a reported maximum P sorption capacity of around 9 mg P $g^{-1}$ d.w. (Ross et al., 2008; Spears et al., 2013). Suppliers have proposed this material as a remediation tool in U.K. lakes, and the effects of dosing on sediment P properties and the impacts of the application on natural sediments are examined by Meis et al. (2013, 2012). Several studies emphasize the Phoslock™ effectiveness in control eutrophication in lakes (Bishop et al., 2014; Epe et al., 2017; Lürling and Oosterhout, 2013), but the sediment smothering of benthos is noticed as a potential adverse effect of this remediation technique (Hickey and Gibbs, 2009). Furthermore, previous research into the use of lanthanum for reducing phosphates has revealed that this natural element can be toxic for the aquatic communities, depending on the concentration and the rate of application (Douglas et al., 2004). Even Martin and Hickey (2004) classified Phoslock™ as a no hazardous material, La ions could be toxic to some aquatic species, particularly for algae and cyanobacteria (González et al., 2015; Jin et al., 2009), cladocerans such as *Daphnia* (Barry and Meehan, 2000; Herrmann et al., 2016), and also for some soil invertebrates species (particularly isopods and earthworms) (Li et al., 2018). On the contrary, some other studies highlighted the innocuous effect on *Daphnia* (Lürling and Tolman, 2010) and on chironomid larvae (Waajen et al., 2017) when Phoslock™ and its active ingredient lanthanum are applied in eutrophication control.

Finally, the use of natural modified zeolites are the more recent approach used in the water remediation and presently it represents the only active sediment capping agent that targets both N and P (Hickey and Gibbs, 2009). A natural zeolite is an abundant and

readily available resource, is an aluminosilicate material commonly occurring in sedimentary rocks, and present a large specific adsorptive surface area because of its fine porous structure (Zamparas and Zacharias, 2014). Natural zeolites possess a net negative charge and this means that they have an affinity for cations, such as ammonium ($NH_4^+$) (Wen et al., 2006). For this reason, natural zeolites are used in water remediation, particularly for the removal of cationic pollutants (Reeve and Fallowfield, 2018; Wang and Peng, 2010). However, to make this material suitable for broader applications, it is possible to modify the chemical properties of their particle's surface by several methods, such as ion exchange with alkaline basis, acid-steam and/or high temperature treatments, and aluminium addition (Ates and Akgül, 2016; Ates and Hardacre, 2012; Taffarel and Rubio, 2009). The resultant modified zeolite, thus become capable to adsorb cations, anions and/or non-polar organic molecules from water (Bowman, 2003). In fact, numerous pollutants that possess an anionic charge, as the case of many viruses and bacteria can be removed from water and wastewater by specific modified zeolites (Bowman, 2003; Y. Li et al., 2016; Reeve and Fallowfield, 2018; Tran et al., 2018). Related to the suitability of modified zeolites for in lake remediation applications, there are several studies highlighting the success of the modified zeolite Z2G1 (with a thin layer of 2 mm) in removing P from sediment under aerobic and anaerobic conditions (Gibbs and Özkundakci, 2011; Zamparas and Zacharias, 2014). Furthermore, Yang et al. (2014) showed that zirconium-modified zeolites added into the lake sediment can reduce the release of phosphate-P from the sediment as well increase the phosphate-P adsorption capacity of the sediment. Although the lack of information regarding zeolite's ecotoxicity, the modified zeolite hexadecyltrimethylammonium (HDTMA) (Reeve and Fallowfield, 2018) and the surfactant-modified zeolite (Li and Bowman, 1998) did not showed toxicity for microorganisms, while the commercial zeolite A displayed a genotoxic effect on *Acinetobacter junii* and *Saccharomyces cerevisiae* (Hrenović et al., 2010). As a part of the product registration process for a lake treatment, Martin and Hickey (2007) assessed the toxicity of modified zeolite Z2G1 to sediment-dwelling species (the amphipod *Phreatogammarus helmsii* and the clam *Sphaerium novaezelandiae*) at a range of modified zeolite doses. The results of sediment toxicity tests (10 days exposure) showed a

significant impact on clam reburial at 700 g $m^{-2}$ and amphipod survival at 2100 g $m^{-2}$, with no detectable effects for *Daphnia magna* or other clams at the proposed lake treatment dose of 350 g $m^{-2}$.

An *in situ* exposure, the same product was used into Lake Okaro (New Zealand) at a nominal concentration of 350 g $m^{-2}$, and there were no impacts on the survival of freshwater crayfish (*Paranephrops planifrons*) and any significant sub-lethal effect was observed on their mobility or physiology (Parkyn et al., 2011). According to these authors, applications of 350 g $m^{-2}$ modified zeolite Z2G1 will have no short-term effect on adult crayfish, with a "margin of safety" for higher application rates.

Before the implementation of an active management regime for controlling internal nutrient loads, an Ecological Risk Assessment is required to address potential the adverse effects. Considering that physical and chemical approaches can have significant benefits in reducing the proliferation of cyanobacterial blooms, other potential adverse ecological effects may occur. For this reason, the costs, the application strategy, the potential stressor(s) effect(s) both for humans and key species, are factors that should be taken into consideration before its effective application in the filed. An evaluation can be made by taking advantage of microcosm and mesocosm experiments to mimic, as much as possible, the systems under evaluation.

### 1.5. Goals and thesis structure

The need to reduce anthropogenic nutrient inputs to aquatic ecosystems, aiming to protect drinking-water supplies and also to reduce eutrophication has been widely recognised. In developed countries, the intensive agriculture and industrial production have been identified as the most critical threats to surface and groundwater resources, by the diffuse pollution with nutrients, pesticides and metals. In fact, soil contaminants can enter the water bodies through leaching or surface and sub-surface runoffs, and the complex interactions between soil and aquatic compartments should be considered to evaluate the ecological integrity of freshwater ecosystem.

In Europe, one of the most important legal instruments in force is presently the Water Framework Directive 2000/60/EC (EU-WFD), whose main goal is to achieve a good

ecological status by the establishment of a framework for evaluation of inland surface waters, transitional waters, coastal waters and groundwaters. At a national level, the ecological state of lagoons and shallow freshwater lagoons has been documented in the last decades, and several regulatory measures were applied aimed at reducing external nutrient loadings and in restoring and/or protecting affected aquatic ecosystems. Although the first approach to control eutrophication is primarily related to the managing of external loading of nutrients, other complementary approaches are needed to regulate the release of nutrients from bottom sediments to the water column. These measurements may include physical, chemical and/or biomanipulation methods, which may represent a potential environmental remediation strategy for eutrophic freshwater systems.

Thus, the present thesis is structured into five chapters, the first and fifth chapters concern to the general introduction and to the main conclusions and final remarks of the thesis, respectively. The general introduction was already accepted for publication in an ISI-WOS scientific journal. The other three chapters are related to research work and were also published in ISI-WOS scientific journals. Below is a brief description of the objectives for each chapter.

**[Chapter II – Effects of dietary exposure to herbicide and of the nutritive quality of contaminated food on the reproductive output of *Daphnia magna*]**

The objective of this chapter was to assess how the exposure to the herbicide pendimethalin (Prowl®), under environmentally relevant concentrations, may compromise the nutritional composition of food for a relevant group of primary consumers of freshwater food webs – the daphnids, thus affecting their reproduction performance and subsequently the long-term sustainability of active populations of this grazer organism. For this evaluation, *Daphnia magna* individuals were chronically exposed in a clean medium to a control diet (NCF – i.e. non-contaminated green algae *Raphidocelis subcapitata*) and to a contaminated diet (CF – i.e. the same monoalgal culture grown in a medium enriched with pendimethalin in a concentration equivalent to the $EC_{20}$ for growth inhibition of algae), during which reproductive endpoints were

assessed. The algae were analyzed for protein, carbohydrates and fatty acid content, and the bioaccumulation of pendimethalin by *D. magna* was also evaluated.

**[Chapter III – Soil ecotoxicological screening (Tier 1) for a diffuse-contaminated drainage area surrounding a lacustrine ecosystem in the Centre of Portugal]**

This chapter aimed to assess a different approach for the application of the Dutch Risk Assessment Framework, developed for contaminated sites, to areas undergoing diffuse pollution from agricultural activities. Our approach consisted in use the ecotoxicological line of evidence (EcotoxLoE) to select the soils for chemical analysis of potential contaminants, and subsequently for an integrated evaluation of risks by combining both the chemical line of evidence (ChemLoE) and EcotoxLoE. This assessment included a battery of cost-effective and time-effective standard bioassays, considering soil habitat function (whole soil approach – Microtox® test and avoidance assays with *Folsomia candida*) and soil retention function (elutriate approach – growth inhibition test with *Raphidocelis subcapitata*) for evaluating a vast array of samples collected in the study area. Considering that analytical methods are extremely expensive and that their use could limit the application of the ERA framework, on a routine basis, to extensive areas affected by diffuse pollution, the approach proposed in our study intend to provide a feasible evaluation for tier 1, with lower costs.

**[Chapter IV – $TiO_2$ nanoparticles for the remediation of eutrophic shallow freshwater systems: Efficiency and impacts on aquatic biota under a microcosm experiment]**

The objective was to investigate, under a microcosm experiment, whether nano-$TiO_2$ affects the contribution of sediment for internal loading of phosphorus, and to what extent chemical properties of nano-$TiO_2$ promote algal aggregation, mitigating algal blooms. Additionally, this study aimed to assess if nano-$TiO_2$ may affect freshwater biota and their populations when applied for environmental remediation purposes in eutrophic systems. For this, the macrophyte *Lemna minor* (growth inhibition endpoint) and the cladoceran *Daphnia magna* (reproduction and growth endpoints) were used as

representative planktonic species, and *Chironomus riparius* (growth inhibition endpoint) was chosen as a representative benthic species for ecotoxicological assays.

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# Chapter II

## Effects of dietary exposure to herbicide and of the nutritive quality of contaminated food on the reproductive output of *Daphnia magna*

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# Effects of dietary exposure to herbicide and of the nutritive quality of contaminated food on the reproductive output of *Daphnia magna*

**Abstract**

Risk assessment of pesticides has been based on direct toxic effects on aquatic organisms. Indirect effects data are taken into account but with limitations, as it is frequently difficult to predict their real impacts in the ecosystems. In this context, the main aim of this work was to assess how the exposure to the herbicide pendimethalin (Prowl®), under environmentally relevant concentrations, may compromise the nutritional composition of food for a relevant group of primary consumers of freshwater food webs – the daphnids, thus affecting their reproduction performance and subsequently the long-term sustainability of active populations of this grazer. Therefore, *Daphnia magna* individuals were chronically exposed in a clean medium to a control diet (NCF - i.e. non-contaminated green algae *Raphidocelis subcapitata*) and to a contaminated diet (CF - i.e. the same monoalgal culture grown in a medium enriched with pendimethalin in a concentration equivalent to the $EC_{20}$ for growth inhibition of algae), during which reproductive endpoints were assessed. The algae were analysed for protein, carbohydrate and fatty acid content. The chemical composition of *R. subcapitata* in the CF revealed a slight decrease on total fatty acid levels, with a particular decrease of essential ω9 monounsaturated fatty acids. In contrast, the protein content was high in the CF. *D. magna* exposed to CF experienced a 16% reduction in reproduction, measured as the total number of offspring produced per female. Additionally, an internal pendimethalin body burden of 4.226 μg $g^{-1}$ was accumulated by daphnids fed with CF. Hence, although it is difficult to discriminate the contribution of the pesticide (as a toxic agent transferred through the food web) from that of the food with a poor quality compromised by the same pesticide, there are no doubts that, under environmentally relevant concentrations of pesticides, both pathways may compromise the populations of freshwater grazers in the long term, with consequences in the control of the primary productivity of these systems.

**Keywords:** Pendimethalin; *Raphidocelis subcapitata*; Nutritional quality; Freshwater trophic relations

## 2.1. Introduction

According to the Millennium Ecosystem Assessment, agriculture has been one of the main driving forces in the degradation of aquatic systems in the last 50 years, especially due to the overuse of fertilizers and pesticides (MEA, 2005). Although the dominant route of pesticide exposure for aquatic species is through direct uptake from water column, organisms may also be exposed to toxic levels of pesticides via food ingestion (Barata et al., 2002; Komjarova and Blust, 2009; Muñoz et al., 1996; Rahman et al., 2012), with subsequent direct and indirect effects.

In most aquatic ecosystems, phytoplankton is a key group providing energy for the survival of primary consumers, both invertebrates and vertebrates (Ma et al., 2002; Prado et al., 2009), being the main source of (high quality) food (Brett et al., 2009). Primary consumers assimilate organic and inorganic compounds from their diet for biosynthetic reactions, somatic growth, reproduction and repair, but they also incorporate contaminants (Kainz et al., 2008), with risks to higher trophic levels of the food chains. Cladocerans of the genus *Daphnia*, primary consumers in freshwater food webs, have been reported as important players in the control of eutrophication, due to their high grazing activity on the phytoplankton (Hansson et al., 2004) including less edible forms like cyanobacteria (Hansson et al., 2004; Peretyatko et al., 2012, 2009). However, these food web interactions could be affected by the quality of phytoplankton as a main food source (Danielsdottir et al., 2007). A high food-quality alga allows the zooplankton community to sustain relatively high biomass and to depress phytoplankton biomass to low levels (Danielsdottir et al., 2007).

Up to now, polyunsaturated fatty acid (PUFA) (Gladyshev et al., 2011; Li et al., 2002; Taipale et al., 2011), sterol content (Martin-Creuzburg and Von Elert, 2004; McLarnon-Riches et al., 1998), and the elemental C:P ratio (De Schamphelaere et al., 2007; Müller-Navarra, 1995) of algae have been considered important biochemical indicators of food quality for *Daphnia*. PUFA are essential for growth and development of organisms, as

they are vital components of cell membranes, playing essential roles in cell function and regulation (Gulati and Demott, 1997).

Nutritional quality of food for primary consumers varies with different factors (e.g. species, environmental factors, trophic status of the system) (Brett et al., 2006; McLarnon-Riches et al., 1998). Besides, several authors have also emphasized the impacts of contamination on inorganic carbon integration and in the biochemical composition of algae (Evens et al., 2009; McLarnon-Riches et al., 1998). Some other studies have focused on how algal blooms affect the accumulation of some inorganic contaminants in *Daphnia* sp. after a period of grazing (Chen et al., 2007; Pickhardt and Fisher, 2007; Pickhardt et al., 2002). These authors reinforced that a potential interaction between algae and contaminants could have important influences on the actual bioaccumulation and trophic transfer of harmful substances in aquatic food webs.

Although the effects of contaminants on food quality for cladocerans have been investigated to some degree, only few studies have investigated the potential implications of pesticides on food quality (Kent and Caux, 1995; Weiner et al., 2007). Hence, more research studies are needed, especially for a more diverse array of pesticides, and especially for commercial formulations, which are the ones applied in the field, and whose toxicity could be different from that recorded for their active ingredients (Pereira et al. 2009).

Pendimethalin, a commonly used pesticide to control annual grasses and certain broadleaf weeds, is known to be highly toxic to non-target organisms, especially to aquatic species (USEPA, 1997). However, little can be found in the literature about both accumulation and toxicity of pendimethalin.

In this context, we hypothesized that the effects induced by this pesticide on primary consumers are caused by both its chemical toxicity and changes in food quality it causes. Therefore, the aims of the present study were: i) to evaluate changes in the nutritive quality of the phytoplanktonic species *Raphidocelis subcapitata*, previously exposed to sub-lethal concentrations of the pesticide Prowl® (a.i. pendimethalin); ii) to assess changes on the growth and reproductive output of *D. magna* fed with

*R.subcapitata* previously exposed to sub-lethal concentrations of Prowl®, and iii) to evaluate the bioaccumulation of pendimethalin by *D. magna.*

## 2.2. Material and methods

### 2.2.1. Pesticide properties

Prowl®, the pesticide used in the present study, is a commercial herbicide formulation with an active concentration of 300 g $L^{-1}$ pendimethalin (BASF, Portugal).

Pendimethalin (N-(1-ethylpropyl)-2,6-dinitro-3,4-xylidine), a dinitroaniline herbicide, has the empirical formula $C_{13}H_{19}N_3O_4$ and the CAS no. 40487-42-1. This herbicide is widely used before crop emergence or planting, inhibiting the stages of plant cell division responsible for chromosome separation and cell wall formation (Ündeğer et al., 2010). According to the US Environmental Protection Agency, pendimethalin is slightly or moderately persistent in the environment and may contaminate surface and groundwater (USEPA, 1997). The widespread use of pendimethalin in various formulations has led to that it can be detected in soil, groundwater, surface water and air (Danion et al., 2012). In freshwater, pendimethalin concentrations reach 6 μg $L^{-1}$ after water runoff, while concentrations of 8 μg pendimethalin $L^{-1}$ have been recorded in more enclosed systems (Strandberg and Scott-Fordsmand, 2004). Also, pendimethalin levels of 17.6 μg $L^{-1}$ were found at natural surface water (USEPA, 1997).

### 2.2.2. *Raphidocelis subcapitata* and *Daphnia magna* culture conditions

*Raphidocelis subcapitata* was obtained from a culture reared in the laboratory. The cultures were maintained under controlled temperature (20 ± 2ºC), photoperiod ($16h^L$: $8h^D$) and light intensity (60-120 μE· $m^{-2}$ $s^{-1}$) conditions. To start new cultures, algae were harvested during their exponential growth phase (6-7 days old) and inoculated in a fresh medium.

*Daphnia magna* (clone A) was obtained from a laboratory stock culture. Organisms are continuously maintained in synthetic ASTM hardwater medium (ASTM, 1996) supplied with an organic additive extracted from the alga *Ascophyllum nodosum* (Baird et al., 1989), at constant temperature (20 ± 2ºC), photoperiod ($16h^L$:$8h^D$), and light

intensity not exceeding 15-20 $\mu E \cdot m^{-2} \cdot s^{-1}$. *D. magna* culture medium is renewed every other day and the organisms were fed with *R. subcapitata* at a rate of 3.0 x $10^5$ cells $mL^{-1}$ $day^{-1}$.

### 2.2.3. Experimental design

The green algae of the species *R. subcapitata* were cultured in MBL control medium to give rise to non-contaminated food (NCF) and in MBL medium containing a concentration of pendimethalin equivalent to the $EC_{20}$ value for algal growth inhibition to obtain contaminated food (CF) (see Section 2.2.4). The algae were harvested and their nutritional value was determined in terms of protein, carbohydrate and fatty acid content (see section 2.2.7). Both NCF and CF algae were used as food to feed *D. magna* during chronic assays (see Section 2.2.5).

### 2.2.4. *R. subcapitata* growth inhibition assay

Algal growth inhibition assay with *R. subcapitata* was conducted according to the OECD 201 guideline for the testing of chemicals (OECD, 2006). A range of concentrations of pendimethalin arranged in a geometric series with a factor of 1.8 (0.294, 0.529, 0.953, 1.715, 3.086, 5.556 and 10.000 $\mu gL^{-1}$) was defined. Test solutions of the chosen concentrations were prepared by mixing a stock solution of the pesticide with Woods Hole MBL growth medium. The exposures were performed in 100-mL glass vials filled with 40 mL of the test medium, using a total of three replicates per each treatment plus controls consisting of MBL culture medium only. Test vials, with an initial cell density of ~$10^4$ cells $mL^{-1}$ (inoculated from the exponentially growing batch culture), were incubated under continuous agitation (~100 rpm in an orbital shaker) and in the same conditions of algal cultures for 96h (see Section 2.2.2). At the end of the bioassay, the cell density was determined using a Neubauer counting chamber, and the growth rate ($day^{-1}$) was determined. In order to calculate the effect concentration causing a 20% ($EC_{20}$) growth inhibition and its respective 95% confidence limits, a non-linear regression using the logistic model was fitted to the data using IBM SPSS.19.

### 2.2.5. Food preparation

In order to obtain contaminated food (CF) to feed *Daphnia magna*, *R. subcapitata* was cultured under the conditions previously described in Woods Hole MBL containing a concentration of pendimethalin corresponding to the $EC_{20}$ for growth rate previously determined (7.58 µg $L^{-1}$) through the growth inhibition assay (see Section 2.2.4). Culture medium without pendimethalin was used to obtain non-contaminated food (NCF). Fresh food for the experiments (CF and NCF) was prepared weekly. Algal growth ended after 6-7 days of exposure and algae from each culture were harvested by centrifugation at 4000 rpm for 4 min. Supernatant was discarded and the remaining pellets in each flask were washed twice with ASTM medium, after which a part of them was separated for biochemical analysis (see Section 2.2.7), whereas the remaining algae served as food to feed daphnids during the chronic assays (see Section 2.2.6). Samples for lipid analysis were freeze-dried and samples for protein and carbohydrate analysis were stored at -20ºC until analysis.

### 2.2.6. Chronic bioassays with *D. magna*

Chronic assays with *D. magna* were conducted according to the OECD guideline No. 211 for the testing of chemicals (OECD, 2012) with some adaptations. At the beginning of the experiment, ten neonates born between 3$^{rd}$ and 5$^{th}$ broods, less than 24h old, were transferred individually into a glass beaker containing fifty milliliters of synthetic ASTM hardwater medium (ASTM, 1996). For each treatment (NCF and CF), ten replicates were used. During the experiment, organisms were kept under the conditions described for culture maintenance. Dissolved oxygen and pH were monitored throughout the test for validation purposes. Every other day, the medium was completely renewed and the organisms were fed with *R. subcapitata* at a rate of 3.0 x $10^5$ cells $mL^{-1}$ $day^{-1}$, while reproduction was recorded daily as the number of offspring released from the brood pouch. The offspring were counted and removed. Survival was assessed daily, whereas the body length of the females – estimated by extrapolation from the exopodite length of the moulted enxuvia (Pereira et al. 2004) – was determined at the beginning and at the end of the assay, allowing the calculation of the somatic growth rate. The age at the first

reproduction was also recorded. Ten replicates consisting of one adult female were used per treatment. The tests were conducted until the fifth clutch of *D. magna* has been released in both NCF and CF treatments (corresponding to 26 days of exposure).

### 2.2.7. Biochemical composition of *R. subcapitata*

Algal samples weekly prepared from each culture (NCF and CF conditions) were measured for protein, carbohydrate and fatty acid content (3 replicates for each content, per each flask, per week, for both NCF and CF, a total of 72 replicates).

For carbohydrate and protein analysis, 1 and 2 mL of resuspended pellet per sample were used, respectively. Algal pellets were digested overnight in 0.1 N NaOH at 4ºC, centrifuged at 14000 rpm for 10 min, and supernatants were used for protein and sugar analysis according to Bajguz and Koronka (2001) procedure. The absorbance of carbohydrate and protein extracts were measured spectrophotometrically and transformed into concentration equivalents using the cell density per milliliter in each measured sample. The Lowry method was used for protein determination (Lowry et al., 1951). Soluble carbohydrates were measured according to the phenol/sulphuric acid colorimetric method outlined in Dubois et al. (1956). Percent soluble carbohydrate was calculated based on absorbance values obtained at 490 nm in a spectrophotometer after obtaining a calibration curve using glucose as standard. Biochemical composition of food (regarding protein and carbohydrate content) was calculated from the cell density measurements. Number of cells was determined by direct counting cells in both NCF and CF growth mediums using the Neubauer chamber.

Algae for fatty acid analysis were concentrated by centrifugation (15 min at 4000 rpm), lyophilized and stored at -20ºC until further use. Fatty acids were converted into their methyl ester derivatives by direct transesterification of freeze-dried samples, according to the acidic method described by Lepage and Roy (1984) with modifications introduced by Cohen et al. (1988) using heptadecanoic acid as internal standard. The analysis of those esters was carried out using a gas-liquid chromatographic system (Hewlett Packard 5890 GC), equipped with a flame ionisation detector and a polar WCOT-fused silica capillary column (50 m length, CP-Sil 88, Chrompack, The Netherlands). The

oven temperature was programmed to increase from 170 to 220ºC, at a rate of 1ºC $min^{-1}$. Pure standards (Sigma) were used for fatty acid identification, which was based on a comparison of peaks retention times between samples and standards. Peak areas were quantified with an HP-3395 integrator, and calculations were performed according to the AOCS Official Method Ce 1b-89 (Firestone, 1994).

### 2.2.8. Pendimethalin residues in adult females

To measure the residues of pendimethalin in adult female organisms, after the chronic exposure to CF and NCF in clean test ASTM medium, a significant amount of wet biomass was needed. For this purpose, more than a thousand organisms, obtained from the same broods as the organisms used in the assay previously described (see Section 2.2.6) were used per treatment (CF and NCF), and kept in parallel under the same conditions of test organisms, to ensure enough biomass for the analysis. At the end of the experiment, the adult females were washed twice with clean medium to remove the external body residues and then placed in Eppendorf tubes and stored at -20ºC for later determination of pendimethalin. One gram of daphnids (wet weight) was placed in a glass thimble (35 mm x 90 mm; 40-60 mm porous disk) for a Soxhlet (extractor volume 100 mL). The extraction was performed for 24 h with methanol (4 cycle's $h^{-1}$). The organic layers from the Soxhlet extractions were dried through 5 g of anhydrous sodium sulphate, washed with hexane and concentrated by evaporation with a rotavapor. The residue was transferred with 1 mL of hexane on top of a glass chromatography column equipped with a stopper. The column was filled as follows: first a plug of glass wool, followed by 2 g Florisil 100-200 mesh and 2 g anhydrous sodium sulphate. Florisil was first activated at 650$^{o}$C, for 3 h, and then deactivated with deionized water. The column was eluted with 20 mL hexane:methylene chloride 9:1 followed by 50 mL hexane:methylene chloride 8:2. The eluate was collected in a 100 mL conical flask. The extract was evaporated to dryness and kept until GC-MS analysis.

One microliter of each sample was injected into GC-MS Shimadzu QP5000 Ex equipped with a capillary column DB 5MS 60m, 0.2 mm, 0.32 from J&W Scientific. The temperature program was as follows: 1 min at 80ºC, then a progressive increase to 285$^{o}$C

at a rate of 20°C/min and finally 43.75 min at 285°C. The GC-MS was operated in single ion monitoring mode SIM, the signal intensity for MZ 252.10 and 253.05 was measured for pendimethalin. The concentration of pendimethalin was determined by direct interpolation in the standard curve within their linear dynamic range, and the detection limits were calculated using $y = y_B + 3s_B$, where $s_B$ is the SD of the blank signal estimated as $s_{y/x}$, the residual SD was taken from the calibration line, and $y_B$ is the blank signal estimated from the intercept taken also from the calibration line (Miller and Miller, 2005).

#### 2.2.9. Statistical analysis

Differences between the content in proteins, carbohydrates and fatty acids of *R. subcapitata* exposed to pendimethalin (CF) and non-exposed specimens (NCF) were evaluated with one-way analysis of variance (ANOVA), after checking for assumptions. The same procedure was used to test for differences in *Daphnia* parameters (reproductive endpoints and somatic growth rate) between organisms exposed to CF and NCF. The significance level of all statistical analyses was set at 0.05.

### 2.3. Results

#### 2.3.1. Biochemical composition of phytoplankton

A significantly increased protein content was recorded in algae grown in the presence of pendimethalin (CF) ($F = 11.08$; d.f. = 1,23; $p = 0.003$) (Figure II.1A), however, no significant differences were observed for carbohydrate content caused by the exposure to the pesticide ($F = 0.73$; d.f. = 1,23; $p = 0.402$) (Figure II.1B).

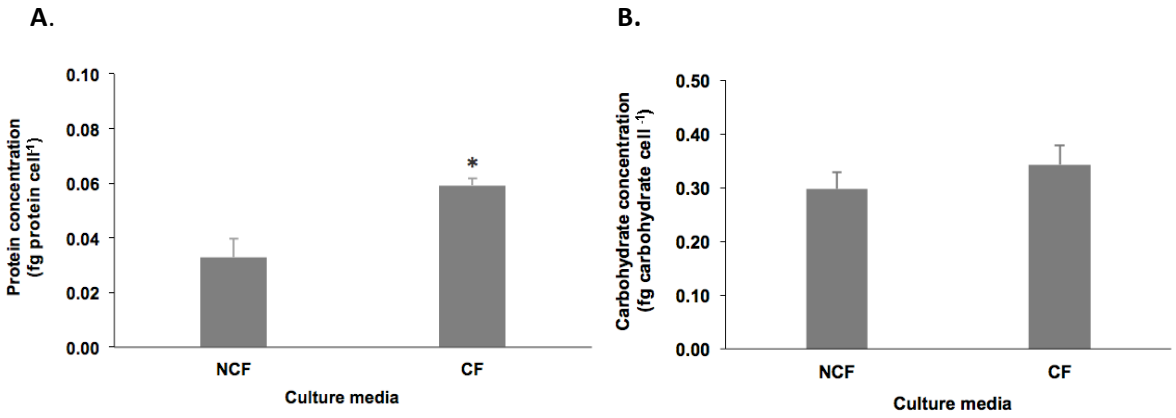


**Figure II.1.** Average values of protein (**A**) and carbohydrate concentration (**B**) (± standard deviation) in *R. subcapitata* grown in both non-contaminated (NCF) and contaminated (CF) culture medium with pendimenthalin.

Overall, with respect to fatty acid composition, *R. subcapitata* was found to contain predominantly $C_{16}$ (16:0) and $C_{18}$ (18:0, 18:1ω9, 18:2ω6 and 18:3ω3) fatty acids, accounting for about 50% of the total fatty acids (Table II.1).

In general, lipid composition was similar in algae fed differently. However, CF algae showed a significant reduction in the content of some specific fatty acids: 11:0 ($F = 5.335$; d.f. = 1,21; $p = 0.032$), 15:1 ($F = 16.02$; d.f. = 1,13; $p = 0.002$), 16:0 ($F = 9.785$; d.f. = 1,16; $p = 0.007$) and 18:1ω9 ($F = 15.89$; d.f. = 1,9; $p = 0.004$). The total fatty acid content was higher in the NCF than in the CF algae (89.62 vs. 77.98 µg $mg^{-1}$ dry biomass), while saturated fatty acids (SFA) values were similar in both groups of algae (27.34 and 24.45 µg $mg^{-1}$ dry biomass in NCF and CF, respectively). The values showing most significant differences concerned the total monounsaturated fatty acids (MUFA) that appeared to be lower on CF algae compared with NCF algae (35.21 vs. 46.68 µg $mg^{-1}$ dry biomass, respectively). Total polyunsaturated fatty acids (PUFA) levels were very similar in both NCF and CF algae (15.60 and 15.02 µg $mg^{-1}$ dry biomass, respectively). ω3 PUFA content was found higher in both (around 11 µg $mg^{-1}$ dry biomass), compared to ω6 PUFA content (about 4 µg $mg^{-1}$ dry biomass).

**Table II.1.** Average content of fatty acids (± standard deviation) recorded in *R. subcapitata* cells exposed to non-contaminated (NCF) and contaminated (CF) culture media. Asterisks (*) indicate statistically significant differences between algae grown in NCF and CF ($p < 0.05$) for each specific fatty acid.

| FA type | FA structure | NCF (µg/mg dry biomass) | CF (µg/mg dry biomass) |
|---|---|---|---|
| SFA | 10:0 | 0.13 ± 0.01 | 0.13 ± 0.02 |
| | 11:0 | 0.34 ± 0.02 | 0.32 ± 0.02 * |
| | 12:0 | 0.01 ± 0.00 | 0.01 ± 0.00 |
| | 13:0 | BDL | BDL |
| | 14:0 | 0.66 ± 0.04 | 0.65 ± 0.05 |
| | 15:0 | 0.02 ± 0.00 | 0.02 ± 0.00 |
| | 16:0 | 9.94 ± 1.10 | 8.65 ± 0.54 * |
| | 17:0 | 2.80 ± 0.09 | 2.81 ± 0.06 |
| | 18:0 | 12.38 ± 0.90 | 13.88 ± 0.63 |
| | 20:0 | 0.02 ± 0.00 | 0.02 ± 0.00 |
| | 21:0 | BDL | BDL |
| | 22:0 | BDL | BDL |
| | 23:0 | BDL | BDL |
| | 24:0 | 1.04 ± 0.17 | 0.96 ± 0.03 |
| MUFA | 14:1 | 0.06 ± 0.02 | 0.07 ± 0.01 |
| | 15:1 | 10.06 ± 1.01 | 8.47 ± 0.28 * |
| | 16:1ω7 | 2.56 ± 0.02 | 2.57 ± 0.01 |
| | 17:1 | 0.70 ± 0.24 | 0.70 ± 0.24 |
| | 18:1ω9 | 33.18 ± 1.08 | 23.60 ± 0.52 * |
| | 20:1ω9 | BDL | BDL |
| | 22:1ω9 | BDL | BDL |
| | 24:1 | 0.12 ± 0.00 | 0.10 ± 0.02 |
| PUFA | 18:2ω6 | 4.32 ± 0.21 | 4.14 ± 0.25 |
| | 18:3ω3 | 11.20 ± 0.76 | 10.77 ± 0.39 |
| | 18:3ω6 | BDL | BDL |
| | 20:2ω6 | BDL | BDL |
| | 20:3ω3 | BDL | BDL |
| | 20:3ω6 | BDL | BDL |
| | 20:4ω6 | BDL | BDL |
| | 20:5ω3 | BDL | BDL |
| | 22:2ω9 | BDL | BDL |
| | 22:6ω3 | 0.08 ± 0.01 | 0.11 ± 0.00 |
| | ∑FA | 89.62 | 77.98 |
| | ∑SFA | 27.34 | 27.45 |
| | ∑MUFA | 46.68 | 35.21 |
| | ∑PUFA | 15.60 | 15.02 |
| | ∑C18 PUFA | 15.52 | 14.91 |
| | ∑ω3 PUFA | 11.28 | 10.88 |
| | ∑ω6 PUFA | 4.32 | 4.14 |
| | ω3 : ω6 | 2.61 | 2.62 |

Abbreviations: BDL, below detection limit; FA, fatty acids; SFA, saturated fatty acids; MUFA, monounsaturated fatty acids; PUFA, polyunsaturated fatty acids.

### 2.3.2. Bioaccumulation potential of pendimethalin by *D. magna*

After 26 days of exposure to contaminated food, Prowl® was significantly bioaccumulated in daphnids fed with CF algae. Organisms fed with CF algae accumulated 4.226 µg $g^{-1}$ wet biomass of pendimethalin, while organisms fed with NCF algae exhibit an amount below the detection limit of the method (<0.005 µg $g^{-1}$ wet biomass).

### 2.3.3. Reproductive output and somatic growth rate in *D. magna*

The bioassay performed fulfilled the validity criteria established by respective guidelines (OECD, 2012). Reproduction in *D. magna* was significantly reduced in daphnids fed with CF algae. Number of neonates born declined from 124 in the average adult females fed with NCF algae to 104 fed with CF algae, corresponding to a decrease of 16% ($F = 30.88$; d.f. = 1,19; $p < 0.001$) (Figure II.2A). On the other hand, time to first brood did not show significant differences between treatments, as well as brood size. No significant differences in the *D. magna* growth rate were recorded between organisms fed with CF and NCF algae ($F = 3.23$; d.f. = 1,19; $p = 0.089$) (Figure II.2B).

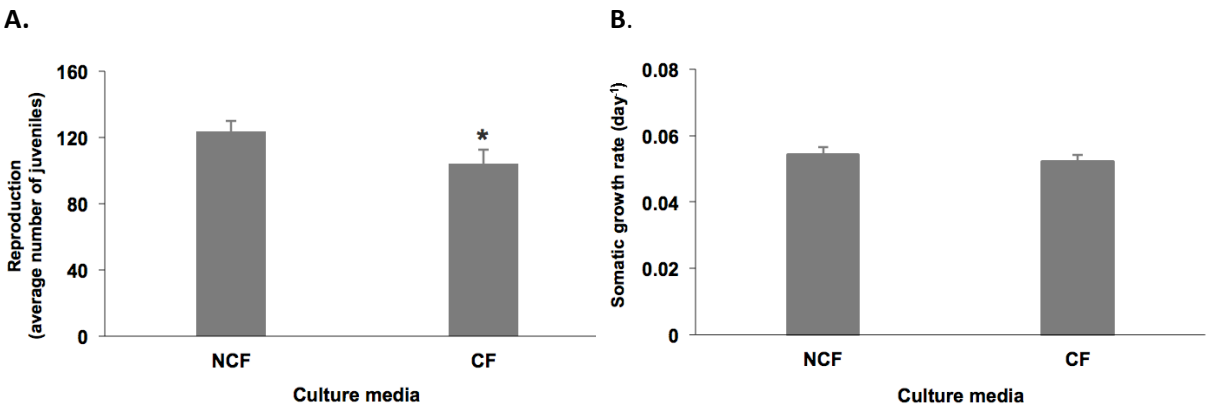


**Figure II.2.** Average number of neonates (**A**) and somatic growth rate (**B**) (± standard deviation) in *D. magna* fed with NCF algae or with CF algae during 26 days of exposure. Asterisks (*) indicate statistically significant differences between daphnids fed with CF and NCF ($p < 0.05$).

## 2.4. Discussion

With regard to the nutritional quality of the algae *R. subcapitata*, our result show that essential LA (linoleic acid, 18:2ω6) and ALA (alpha-linoleic acid, 18:3ω3) fatty acids were equally abundant in algae exposed to both types of medium (LA: 4.32 µg $mg^{-1}$ dry biomass in NCF and 4.14 µg $mg^{-1}$ dry biomass in CF; ALA: 11.20 µg $mg^{-1}$ dry biomass in NCF and 10.77 µg $mg^{-1}$ dry biomass in CF), whereas other PUFA such as docosahexaenoic acid (DHA, 22:6ω3) and eicosapentaenoic acid (EPA, 20:5ω3) were very little synthetized or not detected, respectively. Finding such compositions agree with reports on other chlorophyte species, in which very small amounts of EPA and DHA are synthetized, whereas considerable amounts of LA and some SDA (stearidonic acid, 18:4ω3) (Brett and Müller-Navarra, 1997) are synthetized. ALA is also usually very abundant in green algae, particularly in several species of the genus *Scenedesmus* (Renaudl et al., 1994). Several studies have focused on the impact of low quality food in *Daphnia* performance, as well as finding out which components most interfere their growth and reproduction. According to the earlier studies, diets deficient in some essential fatty acids lead to a decline in *Daphnia* fecundity; particularly when the ingested EPA content is reduced, a decrease in the number of eggs produced is observed (Becker and Boersma, 2005; Wacker and Martin-Creuzburg, 2007). Additionally, many experiments have recognized the nutritional importance of dietary PUFA by using algae supplements to feed daphnids. For example, Martin-Creuzburg and Elert (2009) reared daphnids with different types of food, with main differences in the supplementation or the absence of a specific nutrients (e.g. sterols and PUFA) in the diet composition. They supplemented *S. elongatus* with the fatty acid extract of *Cryptomonas* sp., containing all major ω3-PUFA (ALA, SDA, EPA and DHA) and observed that the enrichment did not improve the somatic growth of *D. magna*, although the availability of dietary ω3-PUFA enhanced egg production. Koch et al. (2009) also reported a strong increase in the number of offspring of *Daphnia galeata* fed with algae supplemented with both lipids and proteins. Based on our study, we can postulate that the inhibitory effect of diet on the *D. magna* reproduction was most likely not related to LA and/or ALA contents. Actually, essential fatty acids LA and ALA are precursors of ω3-PUFA series that can be converted into EPA by daphnids (von Elert, 2002). Nonetheless,

since the conversion rate is low (von Elert, 2002) and the LA and ALA levels are similar in NCF and CF medium, it is unlikely that these differences in LA and ALA could explain the reduction in the reproductive output observed in CF-fed daphnids. In addition, although evidence has been obtained that crustaceans may accumulate EPA and DHA from their food and that these PUFAs support *Daphnia* reproduction (Martin-Creuzburg et al., 2010; von Elert, 2002), the levels measured in both mediums (NFC and CF) were so similar that they probably did not play an important role in the quality of algae used as food for *Daphnia*. On the other hand, oleic acid (18:1ω9) content, found as a major unsaturated fatty acid in some Chlorophyceae species (Desvilettes et al. 1997; McLarnon-Riches et al. 1998; Sahu et al. 2013), showed a significant reduction in algae reared in the contaminated medium (CF). According to Gulati and Demott (1997), supplementing *Synechococcus* diets with an emulsion of oleic acid decreased *D. magna* growth rate, but no effects were reported for reproduction. In this context, the differences found in the levels of oleic acid between the algae reared in NCF and CF media may not as such explain the impairment recorded in the number of offspring in daphnids fed with CF. Regarding the palmitic acid (16:0), a similar prevalence was observed in the two diets (9.94 μg $mg^{-1}$ dry biomass in NCF to 8.65 μg $mg^{-1}$ dry mass in CF), although with a significant decrease in the algae of the CF treatment. According to Ravet et al. (2003), palmitic acid and other ω3-PUFAs interfere in somatic growth and egg production in *Daphnia*, and this saturated fatty acid itself seems to be a limiting factor for *Daphnia* metabolism.

Regarding the protein content of *R. subcapitata* in this study, the herbicide significantly increased its content, and these data are in accordance with Shabana et al. (2001), who reported the rise of protein content in green algae *Protosiphon botryoides* with the increase of pendimethalin concentration. Accordingly, Weiner et al. (2007) showed that the herbicide atrazine induces an increase of protein level in the cyanobacteria *Synechococcus* sp., which may be an indication that metabolic pathways required to synthetize proteins were not affected. Further, it was hypothesized that under stressful conditions it is possible that this particular cyanobacterium produces protein in excess to try to maintain normal cellular function (Weiner et al., 2007).

Carbohydrates are considered to be the most prominent fuel source for *Daphnia*. They fuel the energy demanding of synthetic reactions and provide necessary building blocks for several anabolic pathways (De Coen et al., 2001). Regarding the present study, no statistically significant differences were observed in the total carbohydrate content of algae from NCF and CF medium. Shabana et al. (2001) observed a significant increase of carbohydrate content of the green algae *P. botryoides* with an increase of pendimethalin concentration (from 0.25 mg $L^{-1}$ to 1.0 mg $L^{-1}$), as a common stress response of alga to the pendimethalin injury. In contrast, it was reported that triazine herbicides (gardoprim and terbutryn) at higher concentrations lead to a decrease of the carbohydrate content of *Nostoc muscorum*, which matched the suppression of chlorophyll *a* synthesis; this was attributed to the inhibition of algal photosynthesis (Shabana and Abou-Waly, 1995).

In addition to the indirect effects on *D. magna* reproduction, which might be related to the reduction of nutritive quality of pendimethalin-exposed algae, our study also provides experimental evidence that this active herbicide ingredient had a significant potential to bioaccumulate in daphnids fed with contaminated food, which in turn may compromise *Daphnia* endpoints. Similar trend was observed by Ferrando et al. (1996), who showed that the direct uptake of the insecticide tetradifon in *D. magna* was 2-fold higher, when the daphnids were exposed to water in presence of contaminated food (alga *Nannochloris oculata*) than to contaminated water only, which confirms the ability of algae to cause trophic transfer of contaminants. Moreover, Muñoz et al (1996) fed *Daphnia magna* with *Chlorella* contaminated with pesticide hexachlorobenzene (HCB) for 6 days, and observed that daphnids accumulated 1.7 µg of HCB $Kg^{-1}$ dry weight, showing in parallel a clear reduction (41%) in the number of neonates produced by adults. According to these authors, the major route for pesticide uptake is the consumption of contaminated algae, rather than direct exposure from the water. As concerns pendimethalin contamination, Fliedner (1997) exposed *D. magna* for 48h to both contaminated water and contaminated food, using radioactive-labelled pendimethalin determination. The results showed that daphnids responded more to pendimethalin adsorbed to the food (algae *Scenedesmus subspicatus*) than to pendimethalin dissolved in water. However, these authors only linked the effects observed with the chemical toxicity

of the herbicide. In fact, according to European legislation, this herbicide is highly toxic to both *R. subcapitata* (48h-$EC_{50}$ = 6 µg $L^{-1}$) and *D. magna* (48h-$EC_{50}$ = 280 µg $L^{-1}$ and 21d-NOEC = 14.5 µg $L^{-1}$) (European Commission, 2003).

## 2.5. Conclusions

In general, the reported results suggest that the uptake of pendimethalin through food, at low concentrations of Prowl®, which could be found under real scenarios in agricultural catchments, was clearly responsible for the bioaccumulation of this pesticide and for changes on the reproductive output of *D. magna*. Besides, the tested pesticide reduced the nutritional quality of *R. subcapitata*, especially concerning protein and some fatty acid composition. Hence, both the reduced nutritional quality of food and the accumulation of pesticide through food diet were likely responsible for the observed effects on the reproduction of cladocerans, under real environmental scenarios. Overall, this study underlines the importance of considering both indirect and direct effects of pesticides (e.g. low quality food or bioaccumulation via food uptake) on key freshwater aquatic species, thus increasing the realism of environmental risk assessment (ERA) evaluations.

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# Chapter III

## Soil ecotoxicological screening (Tier 1) for a diffuse-contaminated drainage area surrounding a lacustrine ecosystem in the Centre of Portugal

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# Soil ecotoxicological screening (Tier 1) for a diffuse-contaminated drainage area surrounding a lacustrine ecosystem in the Centre of Portugal

**Abstract**

This study presents a different approach for the application of the Dutch Risk Assessment Framework for contaminated sites, to areas undergoing diffuse pollution from agriculture activities. This approach aims to reduce the costs of tier 1, by using the ecotoxicological line of evidence (EcotoxLoE) to select the soils for chemical analysis of potential contaminants and subsequently for an integrated evaluation of risks by combining both the chemical line of evidence (ChemLoE) and the EcotoxLoE. A battery of cost-effective and time-effective standard bioassays was applied, considering soil habitat function (whole-soil approach – Microtox® test and avoidance assays with *Folsomia candida*) and soil retention function (elutriate approach – growth inhibition test with *Raphidocelis subcapitata*) for evaluating a vast array of samples collected in the study area. After a preliminary calculation of risks based on ecotoxicological data, samples displaying a moderate risk were screened for chemical analysis of the most used pesticides in the area, as well as for total metal concentrations after extraction following standard methods. For these samples, risks based on the ChemLoE and integrated risks were calculated. The ChemLoE confirmed the evaluation made by the EcotoxLoE and reduced the level of risk (<0.5) for the samples formerly presenting a moderate risk. Given the sensitivity of the ecotoxicological assays to the mixture of contaminants potentially found in soils, the approach proved to be a good strategy for the application of the ERA framework, in particular of tier 1, on a routine basis, to areas under diffuse pollution. Since in these areas a more intense sampling is required, it can contribute to reducing the costs of the ChemLoE that can make the application of the ERA framework prohibitive.

**Keywords:** Chemical evaluation; Diffuse pollution; Ecotoxicological evaluation; Integrated risk values; Soil toxicity screening

### 3.1. Introduction

The Millennium Ecosystem Assessment (MEA 2005) gave the world several important insights about the relationship of human societies with natural environments. According to this report, natural ecosystems provide people with a variety of essential services, which include provisioning, regulation, cultural and supporting ecosystem services. Wetlands, in particular, are one of the greatest providers of life support functions, provisioning watering, water supply, fishing, construction materials and fibre production, food crops and livestock grazing, as some other vital resources for human prosperity and wellbeing (Harrison et al. 2010; Kløve et al. 2011). Although wetlands and wetland-dependent ecosystems are protected by a number of EU directives, national legislation and/or international conventions aimed at preserving their biological integrity, several wetlands in Europe are serious threatened (e.g. Paetzold et al. 2010). Diffuse pollution with nutrients, pesticides (or plant protection products in general) and metals has been identified as one of the most critical threats to both surface and groundwater resources (Poissant et al. 2008; Kløve et al. 2011). In developed countries with intensive agriculture and industrial production, wetlands and their living species are under continued high pressure (Eppink et al. 2004). Particularly, agriculture has often been linked to nutrient enrichment or eutrophication of wetlands (van Jaarsveld et al. 2005; Bertahas et al. 2006; Ferreira et al. 2007), and also to contamination with pesticides (Pereira et al. 2009; Iwafune et al. 2010). Related with agricultural areas are frequently livestock activities, which are also responsible for the excessive load of nutrients (especially nitrogen and phosphorus) (Centner et al. 1999; Hooda et al. 2000).

Soil contamination is recognized as a major threat to nearby aquatic systems since through leaching or surface and sub-surface runoff, contaminants can enter the water bodies (e.g. Hooda et al. 2000; Bromilow et al. 2006). Considering the aquatic compartment as the final receptor of the majority of soil contaminants, a more holistic approach is required to evaluate the interactions with the terrestrial environment that may alter their ecological integrity. In this way, to predict the risks of such complex interactions between compartments, terrestrial bioassays should be considered useful tools to diagnose the hazardous of the bioavailable fraction of contaminants in soils

(Alvarenga et al. 2012; Santorufo et al. 2012). To analyse the potential of chemicals to be mobilized and their impact on non-target organisms of different trophic levels, aquatic bioassays with soil elutriates/eluates have been specially recommended (e.g. Maxam et al. 2000; Antunes et al. 2010). In fact, although some authors argue that elutriate procedures tend to underestimate the bioavailable fraction of sorbed compounds (Harkey et al. 1994), its application is crucial to determine the potential mobilization of toxic compounds, or even of salts and nutrients to the aquatic compartment.

Lake Vela, the case study of the present work, is a freshwater resource near Figueira da Foz (Centre of Portugal), which is part of Natura 2000 Site PTCON0055 according to the Resolution of the Council of Ministers 76/2000. During the last 20 years, several studies (Barros 1994; Pereira 1997; Fernandes 1999; Antunes et al. 2003; Abrantes et al. 2006b; de Figueiredo et al. 2006; Castro and Gonçalves 2007) reported the input of nutrients in this aquatic system, which was responsible for the accelerated eutrophication process in the lake. In fact, the slope of the piezometric surface towards the lake depression, the small depth of the groundwater and the existence of small water channels connected to the lake (Fernandes 1999), are site specific characteristics that may enhance the risks of diffuse contamination in the Lake Vela, mainly through leaching and runoff from adjacent agricultural fields (Abrantes et al. 2006b; Abrantes et al. 2008).

An integrated ecological risk assessment (ERA) for Lake Vela has been carried out in the last years, and the studies confirmed the contamination of water and sediment compartments with pesticides more frequently applied in the area (Abrantes et al. 2006b; Abrantes et al. 2009; Abrantes et al. 2010), and also the accumulation of alachlor in predator fish species (Abrantes et al. 2010). Moreover, soil-cores taken from an agricultural field in the Lake Vela surrounds were used to evaluate the ecotoxicological impact of pesticides through leaching on non-target freshwater organisms (Abrantes et al. 2008). This research concluded not only that the primary producers were the most affected trophic level, but also that the current use of pesticides in the lands near Lake Vela, combined with some particular properties of local soils, can contribute to the contamination of surface and groundwater resources. In this context, our study aimed to provide more deep insights to the ERA previously started in Lake Vela, following a more

systematic approach offered by the framework for contaminated sites proposed by Jensen and Mesman (2006). Although few examples are still existing regarding the application of this framework, those available report the application of the scheme to a metal contaminated industrial site (Niemeyer et al. 2010) or to a shooting range (Rodríguez-Seijo et al. 2017). Thus, the novelty of our study is the application of the ERA framework mentioned above to an area undergoing diffuse pollution by plant protection products used in agriculture. For soil risk assessment, a combined procedure including ecotoxicological tests and chemical analysis should be applied to assess contaminated soils. Nevertheless, considering the costs and limitations associated with the chemical analysis of organic contaminants in extensive areas impacted by diffuse pollution, where a representative number of soil samples are required, the main novelty of this study is the proposal of a new approach for tier 1, with a slight modification of the scheme proposed by Jensen and Mesman (2006), in which the ecotoxicological tests are performed first for selecting the samples for subsequent chemical analysis. Hence, chemical analyses could be performed for a more limited number of soil samples, for which risks were identified based on the ecotoxicological line of evidence.

Solid-phase assays are generally closest to real situations (Domene et al. 2008), and organism responses integrate the exposure to the mixture of multiple stressors potentially present in soils. Furthermore, elutriate tests can provide complementary information regarding their potential bioavailability. Based on these previous assumptions, we hypothesize that a battery of whole soil and elutriate tests can firstly reflect the presence of contaminants in the agricultural lands, giving a good indication of those areas where soils may be contaminated to a level that may represent a risk to biota.

For this, a low cost-effective and time-effective battery of bioassays was used to test whole soil samples (the Microtox® solid-phase test with the marine bioluminescent bacteria *Vibrio fischeri* and the avoidance behaviour test with *Folsomia candida)* and soil elutriates (the growth inhibition test with the microalgae *Raphidocelis subcapitata*), following standard protocols. A preliminary risk calculation based on the ecotoxicological

line of evidence was performed in order to scrutinize for the need of further chemical analysis of soils, this time with a reduced number of samples from specific sub-areas.

## 3.2. Material and methods

### 3.2.1. Study area and sampling design

Lake Vela is a eutrophic shallow system located in the littoral centre of Portugal, which occupies 70 ha of floodable surface area. According to Koppen classification (see https://www.ipma.pt/pt/educativa/tempo.clima/index.jsp?page=clima.pt.xml), the lake is included in a region with a temperate Mediterranean climate with mild winters, in which the annual precipitation is of 801-1000 mm and the annual mean temperature is about 15.1-16ºC. Also, summers are characterized as dry and moderately hot, and a long wet period occurred between October and February (Abrantes et al. 2006b).

Forest soils (FS) surround the west side of the lake, whereas the east side is mainly bounded by agricultural and livestock farming soils (AS), as well as human settlements (Abrantes et al. 2008).

The sampling area in the present study was approximately 9 $km^2$, in which sampling points were uniformly distributed throughout the Lake Vela Basin (Figure III.1). Seven transects were defined in north-south direction, considering 500 m of distance between each one, while seven point lines were marked in west-east orientation according to the same distance (500 m) between them. The intersection of multiple transects and multiple point lines gave the final matrix in a total of 46 sampling points, all them georeferenced (Figure III.1). Although FS soils were selected to function as a reference area, since no evidence of historical contamination exists, only some specific FS samples worked as reference soils (for detailed information, see Section 3.2.7). Conversely, points located in the agricultural area (AS) were likely more contaminated, mainly by agricultural and farming practices carried out for several decades (Abrantes et al. 2006b; Abrantes et al. 2008; Abrantes et al. 2009; Abrantes et al. 2010).

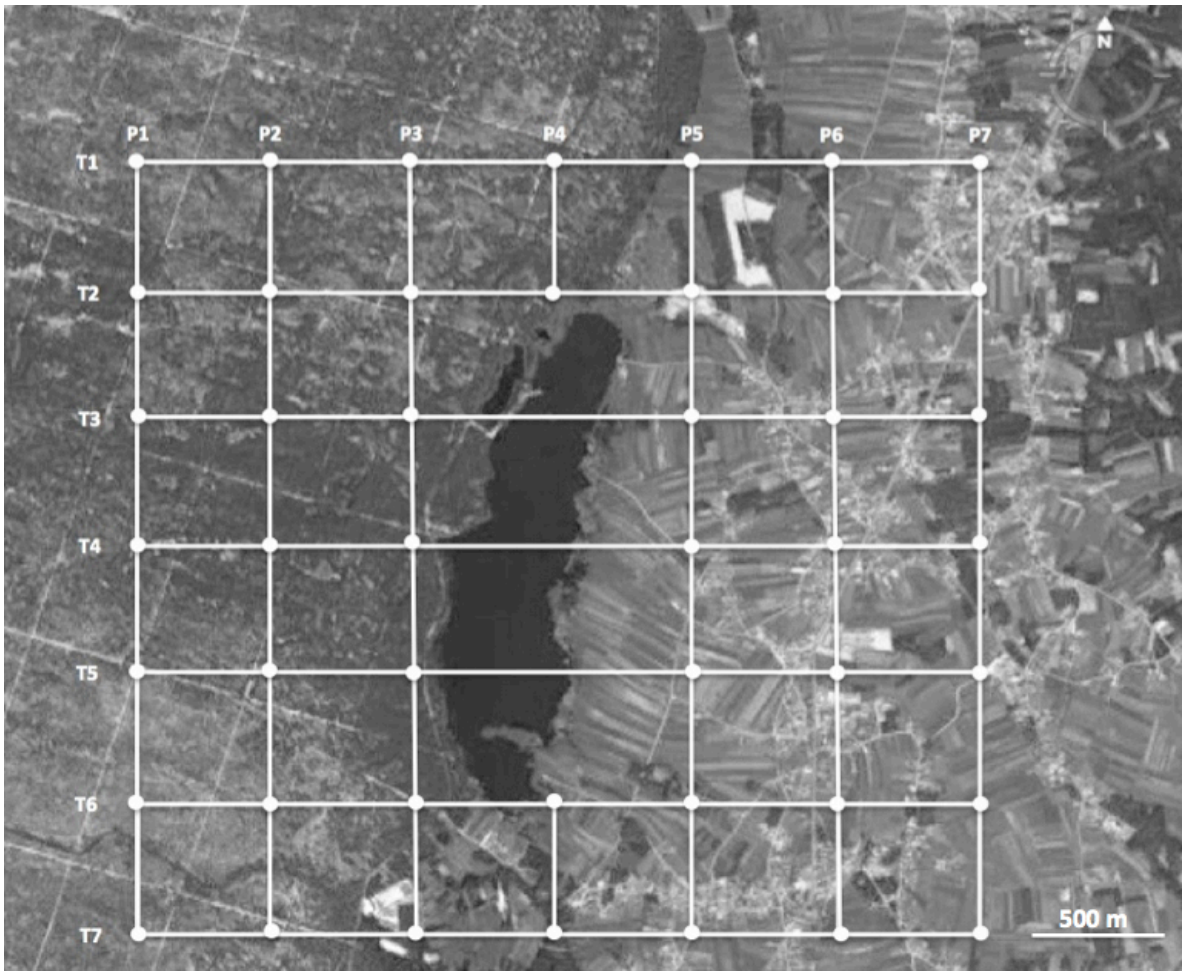


**Figure III.1.** Schematic representation of the study area (Lake Vela system, Figueira da Foz, Portugal) showing the location of the 46 points in the grid defined.

These authors reported the evident contamination of both water and sediment compartments with pesticides, among which alachlor, aldrin, dieldrin, glyphosate and pendimethalin are compounds of special concern for aquatic species (Cerejeira et al. 2003; Antunes et al. 2010; Cruzeiro et al. 2015).

### 3.2.2. Samples collection and storage

The soil sampling was performed in the beginning of autumn, still during the dry season, to specifically address historical contamination. At each sampling point, soil samples were randomly collected from the top 0-20 cm layer, in the area (approximately 9 $km^2$). Samples were collected with a stainless steel corer into clean and opaque plastic bags (for metal and physico-chemical analysis) and wrapped in aluminium foil for the analysis of

pesticides. In the laboratory, soil samples were air-dried and sieved through a 2 mm for soil physical and chemical characterization. For chemical evaluation, the samples were immediately sieved and stored at -20ºC, before analysis. Samples were also sieved through a 2 mm and stored at 4ºC in dark until further use in the ecotoxicological assays, which started in immediately after sampling.

### 3.2.3. Soil chemical and physical characterization

In the laboratory, soil pH and electrical conductivity were determined in a soil/deionised water suspension of 1:5 (w/v) according to an adaptation of the methods described by the ISO guideline 17512-1 (ISO 2008). For each replicate, 10 g of soil were placed in plastic flasks and shaken with 50 mL of deionized water for 30 min. Afterwards, the suspensions were left to rest for 1h, and the pH was measured in the overlying suspension using a pre-calibrated WTW/330/SET-2 pH meter. In the same suspensions, which were left overnight to allow the bulk of the soil to settle, the electrical conductivity was measured using an LF 330/SET conductivity meter. Following the same procedure, soil pH was also measured in a 1:5 (w/v) soil suspension in 1M KCl according to the ISO guideline 17512-1 (ISO 2008). Organic matter content was evaluated by loss of weight of a given portion of dried soil, after ignition at 450ºC, for 8 h (SPAC 2000). Soil maximum water-holding capacity ($WHC_{max}$) was determined according to the guideline ISO 11269 (ISO 2005), as the soil water content after saturation. All previously described physical and chemical parameters were measured in five replicates of soil.

### 3.2.4. Test species and culturing conditions

Lyophilised bioluminescent bacteria (*Vibrio fischeri*), corresponding diluent and reconstitution solution were purchased from Microbics Corporation (Carlsbad, California, USA).

Collembolans (*Folsomia candida* Wilhem) were obtained from a laboratorial culture reared under controlled environmental conditions of temperature (20 ± 2ºC) and photoperiod ($16h^{L}$:$8h^{D}$). A mixture of plaster of Paris and activated charcoal in a ratio of 8:1 (w:w) was used as culture substrate in cylindrical transparent plastic boxes (12-cm

diameter and 6-cm height). Granulated dry baker's yeast was added as food twice a week, in small amounts, to avoid spoilage by fungi. Whenever a new assay was planned, and to obtain synchronized cultures, adult animals were placed on a new culture container to lay eggs. After 2 or 3 days, adults were removed and eggs were left to develop. Juveniles of 10-12 days old were used in avoidance assays. Tests were conducted at the same temperature and photoperiod conditions described for culture maintenance.

The freshwater microalga *Raphidocelis subcapitata* (Chlorophyceae) was obtained from a culture reared in the laboratory. The cultures were maintained in 250-mL Erlenmeyer flasks with 100 mL of Marine Biological Laboratory medium MBL-sterilized Woods Hole Culture, at controlled temperature (20 ± 2ºC) and photoperiod ($16h^{L}$:$8h^{D}$). To start a new culture, algae were harvested from a culture in exponential growth phase (5-7 days old) and inoculated in fresh and sterilized MBL medium.

### 3.2.5. Toxicity assays

#### 3.2.5.1. Assays to test whole soil samples (soil habitat function)

Microtox® was used to evaluate the inhibition in luminescence of the bacteria *Vibrio fischeri* following the basic solid-phase test, originated by each soil sample under evaluation (AZUR Environmental 1998). Thus, suspensions of soils from the Lake Vela area (7g of soil in 35 mL of the basic solid-phase test diluent) were magnetic stirred, for 10 min. An aliquot of 2 mL of the suspension was pipetted and transferred to a test cuvette and then used to make serial dilutions in the Microtox incubation block. After 5, 15 and 30 min of exposure, the bacterial luminescence was measured using a Microtox toxicity analyser model 500 (AZUR Environmental 1998).

The avoidance test with *Folsomia candida* was performed according to the ISO guideline (ISO 2011). The standard artificial OECD soil was prepared according to the guideline 207 and used as control soil in this study (OECD 1984). This soil was composed of quartz sand (70%), kaolin clay (20%) and sphagnum peat (10%) (4 mm sieved), and pH was adjusted to 6.0 ± 0.5 with calcium carbonate. Afterwards, soil moisture and $WHC_{max}$ were determined on five soil replicates following the methodology described above (please see Section 3.2.3).

Before testing, the moisture content of each soil sample was adjusted to 50% of its $WHC_{max}$, through the addition of distilled water. Cylindrical plastic boxes (11.7-cm diameter; 6.2-cm height) were used and divided into two equal sections by means of a card introduced vertically. Thirty grammes (dry weight equivalent) of each soil was placed in one of the sections, while the other one was filled with 30 g (dry weight) of control soil. Five replicates were tested for each soil sample. After removing the plastic divider, 20 organisms (10-12 days old) were placed in the midline of each test chamber. An additional container without organisms was prepared and exposed to the same test conditions, for monitoring pH and moisture at the end of the test. The test containers were closed with perforated transparent lids. At the end of 48-h test period, the two sections of each container were carefully separated and emptied into different vessels, which were filled with water and a few drops of soft ink. After gently shaking, the individuals floating on the water surface were counted. Missing individuals were considered dead. The pH of each soil sample was determined at the beginning and at the end of the test. An additional combination was included containing five dual control replicates, in which control soil was added to both sides of the test container. These replicates were prepared to test for an equal distribution of organisms on both sides of test chambers, when filled with the same soil, thus confirming the good physiological conditions of test organisms.

The calculation of risks was based, in a first step, on the results of soil habitat function tests (Microtox® and avoidance) (for more information, please see Section 3.2.7). When moderate risks were recorded, soil samples were tested for soil retention function by testing corresponding soil elutriates with microalgae (see the following Section 3.2.5.2).

#### 3.2.5.2. Assays to test soil elutriates (soil retention function)

Soil elutriates were prepared as recommended by USEPA (1998a). Erlenmeyer glass vessels were used for preparing a 1:4 [soil (*w*): medium (v)] suspension with Woods Hole MBL medium (Stein 1973). This mixture was shaken for 2h, at 20 ± 2ºC in an orbital shaker (200 rpm), and left to settle overnight, at 4ºC. The overlying aqueous suspension

was decanted and centrifuged at 5000 rpm, at 4ºC, for 15 min and then stored at 4ºC, in the dark, before testing within the next 2 days.

The growth inhibition test with the green algae *R. subcapitata* was conducted according to the OECD guideline no. 201 (OECD 2006), adapted to 24-well microplates (Geis et al. 2000). The algae were exposed during 72-h under continuous illumination to several dilutions of soil elutriates (6.25, 12.5, 25.0, 50.0, 75.0 and 100.0%). Assays were conducted in polystyrene microplates with 6 x 4 flat bottom wells of 1000 μl capacity, using a total of three replicates (wells) per each treatment plus the control (MBL medium). Each well was filled with 900 μl of elutriate (or MBL medium in the CTL) plus 100 μl microalgae inoculum, with an initial cell density of $10^5$ cells $mL^{-1}$, in order to start the test with the same cell's density ($10^4$ cells $mL^{-1}$). Moreover, for each dilution factor, one well was filled with 1000 μl of elutriate (without algae inoculum) in order to exclude any potential interference in the final absorbance induced by the intrinsic colour of the elutriate.

The microp+lates were placed in a climatic chamber under the conditions described for algal cultures. The content of each well was resuspended twice a day by repetitive pipetting to promote active gas exchange and prevent cells clumping. At the end of the bioassay, growth inhibition rate was calculated based on absorbance values obtained at 440 nm in a UV spectrophotometer.

### 3.2.6. Chemical analysis

#### 3.2.6.1. Metals extraction and total metal concentrations in soil samples

The total metal concentrations in the soils classified as moderate risk in the ecotoxicological line of evidence (i.e. T2P5, T3P7 and T6P5), the forest reference soil (i.e. T2P1), and the agricultural sample used to validate the evaluation of risks previously based on ecotoxicological assays (i.e. T5P6) (for more details, please see Section 3.2.7) were microwave digested with nitric acid and hydrochloric acid (3:1), following the method 3051A (USEPA 1998b). The extracts were then analysed by inductively coupled

plasma-mass spectrometry (ICP-MS) for 24 chemical elements including Ag, Al, As, Ba, Ca, Cd, Co, Cr, Cu, Fe, K, Mg, Mn, Mo, Na, Ni, Pb, Sb, Sn, Th, Ti, V, W and Zn.

A rigorous quality control program was implemented including reagent blanks and duplicate samples, and the reference material used was Till1 (Canadian Certified Reference Materials). The recovery results ranged between 83.3% and 110.4%.

#### 3.2.6.2. Extraction and analysis of pesticides from soil samples

Based on farmer's information about the major pesticides used in agriculture near Lake Vela and considering the historic contamination, namely the persistent pesticides that were used in the past, the same soil samples described above (i.e. T2P5, T3P7, T6P5, T2P1 and T5P6) were analysed in an accredited laboratory for the presence of alachlor, aldrin, dieldrin, glyphosate and pendimethalin. The extraction procedures were carried out according to the method no. 3550B (ultrasonic extraction) for soils, published in the SW-846 manual (USEPA 1996). Concentrations of alachlor, glyphosate and pendimethalin were quantified through high-performance liquid chromatography-mass spectrometry (LC-MS/MS), following the methodology described by of USEPA (1999). Finally, concentrations of aldrin and dieldrin were quantified by gas chromatography/mass spectrometry (GC-MS) according to the method no. 8270C (USEPA 1996). Limits of detection ranged between 0.015 and 0.05 mg $kg^{-1}$ depending on the pesticide.

### 3.2.7. Statistical analysis and risk calculation based on the ecotoxicological and chemical lines of evidence

The $EC_{20}$ and $EC_{50}$ (toxic effect thresholds) obtained for *V. fischeri* were computed for each soil using the software MicrotoxOmni® version V1.18 with a linear model (AZUR Environmental 1998). When it was not possible to obtain EC values, the highest percentage of effect was reported.

As for collembolans, the percentage of avoidance for each soil sample was calculated using the following formula:

$$Av\ (\%) = [(C - T)/N] \times 100$$

where Av is the avoidance percentage, *C* is the number of individuals in the control soil (for all replicates), *T* is the number of individuals in the test soil (for all replicates), and *N* is the total number of individuals ( per replicate ).

The Fisher Exact test (one-tail) was used to test for a significant avoidance of each test soil. Data from the dual control replicates were analysed using the same test, but following a two-tailed distribution to test the null hypothesis of equal distribution of organisms between both sides of test containers.

Concerning to growth inhibition test with the algae *R. subcapitata,* one-way ANOVAs followed by the Dunnett multicomparison test were employed to find out potential significant differences in algae growth between the control and tested concentration of elutriates of each soil analysed, using the software SPSS version 21.0 (SPSS Corporation, Chicago, IL). All statistical analyses were run using a significance level set at 0.05.

Risk calculation based on the ecotoxicological line of evidence followed the approach proposed by Jensen and Mesman (2006), where risks were evaluated on a scale ranging from 0 to 1, in which 0 represents “no risk” and 1 represents “high risk”. This method is based on the assumption that the risk of reference soil is zero and that the risk of tested soils is calculated in relation to the value of the respective reference soil (in our case, point P1 from each transect (T) was selected as a reference soil for the other points of the transect). For the ecotoxicological approach, the scaling of the avoidance data was done based on the percentage of avoidance. For the *V. fischeri* tests, the risk was calculated by considering the highest effect recorded on the highest concentration tested for each soil elutriate. Likewise, for *R. subcapitata*, the risk calculation was based on the maximum growth rate inhibition recorded at the highest elutriate concentration tested. Within the ecotoxicological line of evidence (EcotoxLoE), risk values were calculated integrating the data from soil habitat and soil retention functions.

For those sampling points where environmental risks from the EcotoxLoE were moderate, chemical analysis were performed to determine the content of pesticides and metals (for more details, please see previous sections). Thus, risk calculation based on the chemical line of evidence (ChemLoE) also followed the methodology suggested by Jensen

and Mesman (2006), but only taking into account As, Cd, Cr, Cu, Ni, Pb and Zn total concentrations, since the hazard concentration that affects only 5% of the species and microbial processes ($HC_5$) values used for calculating risks based on the ChemLoE were available only for some metals (Jänsch et al. 2007). However, data for the remaining elements analysed is described in the Supplementary Material (Table SIII.1). The reference soil used to calculate the risks for the ChemLoE corresponded to one of the samples from the forest area (i.e. T2P1), randomly selected from the group of P1 samples from each transect T considered as reference soils (please see above), and it is mentioned as FS-REF. Furthermore, another sample with no risk (i.e. T5P6), according to the EcotoxLoE, from AS was also chosen to further validate the evaluation of risks made in the EcotoxLoE.

The risks for the Ecotox LoE were calculated by integrating the data from each assay after it has been scaled and corrected for background levels based on the corresponding reference soil. Regarding the ChemLoE, the toxic pressure of each metal was calculated, and then, the values were integrated to calculate the toxic pressure of the mixture based on the response addition (RA) model. Afterwards, for the five samples analysed for metal content, the risks of both LoEs were integrated and the standard deviation was calculated. The toxic pressure of pesticides was not calculated since they were all found at concentrations below the detection limit.

## 3.3. Results and discussion

### 3.3.1. Soil physical and chemical characterization

Table III.1 shows the general physical and chemical characterization of the 46 test soils. In a general way, the soils from the forest margin (FS) of the lake were acid to neutral ($pH_{H2O}$: 5.32 ± 0.19 to 7.66 ± 0.05; $pH_{KCl}$: 5.02 ± 0.03 to 7.54 ± 0.14), while soils collected in the agricultural area (AS) were slightly more acidic ($pH_{H2O}$: 4.39 ± 0.07 to 7.30 ± 0.06; $pH_{KCl}$: 4.27 ± 0.04 to 7.71 ± 0.02). Regarding the organic matter content, FS showed a low (<2%) organic matter content, while AS generically presented a medium (2 to 6%) organic matter content (USEPA 2004). These characteristics partially agree with those reported by Abrantes et al. (2008), who identified acid values (pH = 5.85) and low to medium organic

matter content in samples from agricultural soils (2.11 ± 0.7%) collected in the area. The low organic matter content observed, combined with the specific properties of local soils, probably have influenced the leaching of chemical compounds and their consequent removal by percolation water, reducing pollutants' availability in the surface layer (Venkata Mohan et al. 2007). Abrantes et al. (2008) also hypothesized that the current use of pesticides in the agricultural lands in the vicinity of Lake Vela could contribute to the contamination of surface and groundwater resources, mainly through leaching, possibly compromising the balance of the freshwater ecosystem.

Concerning to the conductivity, the values from FS presented some variations (ranging from 5.80 ± 0.47 µS $cm^{-1}$ to 62.50 ± 2.92 µS $cm^{-1}$), but with no clear differences in the trend between soils. However, values of AS were comparatively higher (from 25.90 ± 1.04 µS $cm^{-1}$ to 477.00 ± 11.34 µS $cm^{-1}$). In some specific sampling points (T1P6, T3P5, T5P7, T6P4, T5P5), conductivity values were above 235 µS $cm^{-1}$. This increase in conductivity observed in AS was not surprising, and it was possibly correlated with chemical fertilization of agricultural soils in the east margin of the lake (Fernandes 1999; Abrantes et al. 2006a). Furthermore, the sandy nature of soils from Lake Vela drainage and the low content of clay are factors that probably had promoted the excessive use of these agrochemicals in order to achieve profitable agricultural results. Usually, soil salinity is expressed in terms of electrical conductivity of a soil/water suspensions, since it is difficult to measure it in absolute terms of total salts in solution (Malicki and Walczak 1999). Further, salinity is mostly dependent on the water regime, presenting a generic increase during the dry season and a decrease in rainfall phase (Nielsen et al. 2003; Brock et al. 2005), a fact that could in part explain the values obtained in some of our AS samples. In our study, the sampling period took place in October, before the beginning of the rainfall season.

In regards to the water-holding capacity, FS displayed lower values ($WHC_{max}$ < 7% dry mass), while other samples for AS presented a slight increase of water-holding capacity, especially in some points from transects 5 and 6 ($WHC_{max}$: T1P6 = 11.60 ± 1.13; T2P6 = 12.46 ± 1.35; T3P6 = 19.37 ± 1.03; T4P5 = 19.02 ± 1.03; T5P6 = 15.43 ± 2.34). These results were also described by Abrantes et al. (2008), which classified the soils in the

vicinity of Lake Vela as sandy loam soil based on their grain size distribution, a property that contributes for the low water-holding capacity recorded in soils from both east and west margins.

**Table III.1.** General physical and chemical parameters measured in forest soils (FS) and in agricultural soils (AS) from Lake Vela area.

| | Sample | pH ($H_2O$) | pH (KCl) | Conductivity ($\mu S\ cm^{-1}$) | Total organic matter (%) | Water holding capacity (%) |
|---|---|---|---|---|---|---|
| Forest soils (FS) / west margin | **T1P1** | 6.23±0.09 | 6.39±0.10 | 7.94±0.68 | 0.37±0.10 | 5.42±0.51 |
| | **T1P2** | 6.19±0.04 | 5.72±0.02 | 19.83±0.90 | 1.82±0.28 | 5.65±0.14 |
| | **T1P3** | 5.44±0.16 | 5.02±0.03 | 62.50±2.92 | 1.38±0.09 | 5.88±0.51 |
| | **T1P4** | 6.40±0.03 | 6.46±0.04 | 22.50±3.67 | 1.21±0.07 | 6.22±0.57 |
| | **T2P1** | 5.87±0.13 | 5.48±0.05 | 9.66±1.25 | 0.65±0.08 | 3.82±0.67 |
| | **T2P2** | 7.66±0.05 | 6.46±0.05 | 14.04±2.26 | 0.64±0.03 | 6.38±0.63 |
| | **T2P3** | 5.53±0.14 | 5.64±0.02 | 15.30±2.67 | 0.80±0.08 | 7.22±0.57 |
| | **T2P4** | 6.59±0.35 | 6.34±0.06 | 29.20±3.41 | 1.23±0.17 | 6.46±0.48 |
| | **T3P1** | 6.28±0.06 | 6.89±0.03 | 19.66±2.18 | 0.57±0.08 | 3.81±0.58 |
| | **T3P2** | 7.29±0.08 | 6.94±0.02 | 31.17±5.77 | 0.41±0.09 | 5.82±0.30 |
| | **T3P3** | 6.42±0.07 | 5.51±0.09 | 9.24±0.23 | 0.46±0.08 | 5.76±0.33 |
| | **T4P1** | 6.15±0.04 | 6.21±0.04 | 11.28±0.49 | 0.27±0.06 | 3.76±0.32 |
| | **T4P2** | 5.32±0.19 | 5.05±0.03 | 5.80±0.47 | 0.88±0.07 | 3.09±0.20 |
| | **T4P3** | 6.71±0.34 | 5.80±0.06 | 12.20±0.95 | 0.42±0.09 | 4.13±0.36 |
| | **T5P1** | 6.98±0.05 | 7.54±0.14 | 50.96±2.27 | 0.21±0.03 | 5.25±0.84 |
| | **T5P2** | 5.58±0.15 | 5.38±0.03 | 13.03±0.39 | 0.53±0.07 | 9.75±1.33 |
| | **T5P3** | 5.58±0.07 | 5.24±0.04 | 15.97±0.92 | 0.45±0.03 | 2.69±0.34 |
| | **T6P1** | 6.05±0.18 | 5.70±0.02 | 21.84±0.82 | 1.21±0.25 | 5.88±0.17 |
| | **T6P2** | 5.94±0.06 | 5.50±0.03 | 21.08±3.86 | 0.86±0.06 | 9.59±0.87 |
| | **T6P3** | 5.98±0.30 | 5.35±0.05 | 9.53±2.36 | 1.18±0.24 | 3.68±0.38 |
| | **T7P1** | 5.76±0.07 | 5.47±0.05 | 9.14±1.23 | 0.86±0.05 | 2.91±0.77 |
| | **T7P2** | 5.98±0.12 | 5.70±0.07 | 18.18±1.42 | 0.41±0.08 | 3.80±0.57 |
| | **T7P3** | 5.82±0.05 | 5.67±0.06 | 12.68±1.69 | 0.58±0.05 | 2.60±0.24 |
| Agricultural soils (AS) /east margin | **T1P5** | 6.31±0.14 | 6.33±0.05 | 138.67±21.08 | 3.72±0.19 | 7.40±0.68 |
| | **T1P6** | 5.05±0.11 | 5.30±0.01 | 236.60±3.65 | 3.89±0.27 | 11.60±1.13 |
| | **T1P7** | 6.19±0.07 | 6.40±0.06 | 137.32±12.66 | 4.44±0.29 | 9.76±0.78 |
| | **T2P5** | 5.05±0.08 | 4.56±0.02 | 27.10±2.51 | 1.51±0.11 | 3.28±0.29 |
| | **T2P6** | 5.97±0.08 | 6.10±0.01 | 122.15±13.54 | 5.65±0.35 | 12.46±1.35 |
| | **T2P7** | 6.44±0.05 | 6.49±0.04 | 25.90±1.04 | 3.56±0.10 | 4.44±0.35 |
| | **T3P5** | 7.30±0.06 | 7.71±0.02 | 313.75±8.77 | 4.22±0.40 | 6.68±0.75 |
| | **T3P6** | 4.81±0.09 | 5.09±0.03 | 65.67±5.69 | 3.93±0.23 | 19.37±1.03 |
| | **T3P7** | 4.39±0.07 | 4.27±0.04 | 25.60±2.87 | 3.49±0.36 | 5.73±1.54 |
| | **T4P5** | 5.88±0.07 | 6.18±0.07 | 68.14±5.05 | 2.64±0.21 | 19.02±1.03 |
| | **T4P6** | 5.46±0.03 | 5.59±0.03 | 76.20±4.92 | 3.53±0.38 | 8.09±0.93 |
| | **T4P7** | 5.57±0.04 | 5.40±0.09 | 28.20±2.95 | 2.93±0.21 | 4.01±1.36 |
| | **T5P5** | 5.40±0.04 | 5.28±0.02 | 51.80±5.07 | 2.83±0.32 | 4.81±0.93 |
| | **T5P6** | 5.29±0.15 | 5.55±0.08 | 134.00±2.92 | 2.62±0.24 | 15.43±2.34 |
| | **T5P7** | 6.73±0.08 | 7.67±0.08 | 235.50±5.92 | 3.21±0.21 | 8.24±0.11 |
| | **T6P4** | 5.27±0.05 | 5.24±0.02 | 431.00±21.95 | 5.34±0.19 | 10.29±1.18 |
| | **T6P5** | 5.86±0.04 | 5.72±0.01 | 105.20±3.35 | 2.73±0.21 | 4.77±0.45 |
| | **T6P6** | 5.50±0.03 | 5.53±0.06 | 51.24±2.84 | 2.13±0.10 | 4.36±0.05 |
| | **T6P7** | 5.80±0.02 | 5.46±0.04 | 42.66±2.20 | 5.41±0.58 | 5.38±0.52 |
| | **T7P4** | 5.56±0.14 | 5.30±0.02 | 15.34±3.95 | 0.92±0.08 | 4.33±0.75 |
| | **T7P5** | 6.21±0.01 | 6.39±0.04 | 477.00±11.34 | 3.82±0.32 | 6.02±0.72 |
| | **T7P6** | 5.72±0.08 | 5.40±0.01 | 94.10±6.08 | 4.65±0.57 | 5.36±0.65 |
| | **T7P7** | 5.13±0.03 | 5.22±0.01 | 125.18±7.91 | 1.90±0.09 | 3.81±0.49 |

### 3.3.2. Ecotoxicological assays for the evaluation of soil habitat function

Concerning to the soil solid-phase Microtox® assay, the $EC_{20}$ and/or $EC_{50}$ values determined after 5, 15 and 30 min of exposure are summarized in Table III.2, together with the corresponding highest effect (%) for both FS and AS. The classification of samples as toxic was based on the proposal of Kwan and Dutka (1995): samples with values of $EC_{50} \leq 5000$ mg $L^{-1}$ are very toxic; samples with 5000 mg $L^{-1} < EC_{50} \leq 10{,}000$ mg $L^{-1}$ are moderately toxic, and with $EC_{50} > 10{,}000$ mg $L^{-1}$ are non-toxic. Considering the results obtained, no $EC_{20}$ or $EC_{50}$ were computed, for soils from both sides of the lake, since bioluminescence inhibition percentages above 20% were recorded only for the highest dilution/concentration (100%) of elutriates. Nevertheless, regarding the highest inhibition effect (i.e. the maximum inhibition percentage observed in the highest dilution tested, for a 30-min exposure), a value of 48.21% was found for FS (sample T4P2), whereas for AS was 97.68% (sample T3P7) (Table III.2). Generally, according to our data, the bioluminescence inhibition values of the FS revealed a more consistent pattern (ranging from 4.26% to 48.21%), compared to those from AS (ranging from -9.16% to 97.67%). According to the Microtox® test, the results revealed that FS might be characterized as a natural reference area for ecotoxicological evaluations, while for AS soils, data points out for potential soil contamination. In fact, solid-phase Microtox® test is sensible to contaminants presented in the whole soil matrix, and for that reason, this test is usually included in the test batteries for assessing soil retention function (Hund-Rinke et al. 2002; Antunes et al. 2008; Niemeyer et al. 2010; Hentati et al. 2015). In our study, the values related to the bioluminescence inhibition appear to be associated with the substances/contaminants that might be present in the samples, but there are some other parameters that can interfere with the bacteria response. It has been stated that soil properties, particularly the differences in sand and clay content, may give a distinct toxicity pattern for the organisms. Nevertheless, and according to Doherty (2001), *V. fischeri* bacteria can be adsorbed by clay particles that resulted in a reduction of luminescence, but when exposed to sandy soils (with low content of clay) is potentially free in the soil matrix.

**Table III.2.** Effective concentrations (mg $L^{-1}$) and highest effect (HE, %) of soils from forest (FS) and agricultural (AS) area, causing 20% ($EC_{20}$) and/or 50% ($EC_{50}$) inhibition in the bioluminescence of bacterium *V. fischeri* after 5, 15 and 30 min of exposure.

| | Sample | Endpoint | 5 min | 15 min | 30 min |
|---|---|---|---|---|---|
| Forest soils (FS) / west margin | **T1P1** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 16.68 | 19.31 | 25.29 |
| | **T1P2** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 25.48 | 35.40 | 38.96 |
| | **T1P3** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 25.16 | 22.04 | 31.10 |
| | **T1P4** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 12.81 | 17.89 | 22.28 |
| | **T2P1** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 27.29 | 30.78 | 39.76 |
| | **T2P2** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 13.40 | 11.83 | 16.82 |
| | **T2P3** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 12.00 | 16.68 | 17.94 |
| | **T2P4** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 12.86 | 14.14 | 18.45 |
| | **T3P1** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 11.90 | 13.32 | 17.04 |
| | **T3P2** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 7.37 | 9.55 | 12.05 |
| | **T3P3** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 14.63 | 24.41 | 20.93 |
| | **T4P1** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 30.59 | 35.43 | 40.06 |
| | **T4P2** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 30.56 | 38.53 | 48.21 |
| | **T4P3** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 24.37 | 18.83 | 21.16 |
| | **T5P1** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 0.17 | 7.10 | 8.78 |
| | **T5P2** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 27.20 | 35.69 | 39.03 |
| | **T5P3** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 22.29 | 10.71 | 4.26 |
| | **T6P1** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 18.92 | 13.54 | 11.53 |
| | **T6P2** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 15.68 | 7.94 | 4.90 |
| | **T6P3** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 29.65 | 21.90 | 29.18 |
| | **T7P1** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 24.96 | 33.87 | 41.33 |
| | **T7P2** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 13.00 | 17.17 | 22.69 |
| | **T7P3** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 8.42 | 12.59 | 14.79 |

**Table III.2** (continued)

| | Sample | Endpoint | 5 min | 15 min | 30 min |
|---|---|---|---|---|---|
| Agricultural soils (AS) / east margin | **T1P5** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 17.67 | 31.02 | 34.24 |
| | **T1P6** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 39.87 | 24.50 | 25.13 |
| | **T1P7** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 0.50 | -2.72 | 1.31 |
| | **T2P5** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 83.94 | 85.32 | 88.70 |
| | **T2P6** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 23.56 | 20.46 | 27.01 |
| | **T2P7** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 17.56 | 19.76 | 21.50 |
| | **T3P5** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | -24.43 | -23.16 | -9.16 |
| | **T3P6** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 38.02 | 30.51 | 39.16 |
| | **T3P7** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 91.29 | 95.26 | 97.68 |
| | **T4P5** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 26.67 | 38.74 | 39.39 |
| | **T4P6** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 28.30 | 9.63 | 19.05 |
| | **T4P7** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 29.13 | 17.40 | 19.59 |
| | **T5P5** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 27.33 | 20.55 | 23.02 |
| | **T5P6** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 34.68 | 44.00 | 50.62 |
| | **T5P7** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 16.42 | 22.42 | 17.01 |
| | **T6P4** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 34.31 | 30.79 | 38.23 |
| | **T6P5** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 45.21 | 55.01 | 67.25 |
| | **T6P6** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 23.02 | 34.71 | 38.78 |
| | **T6P7** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 16.21 | 26.68 | 25.03 |
| | **T7P4** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 39.20 | 48.58 | 57.78 |
| | **T7P5** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 9.08 | 10.55 | 26.52 |
| | **T7P6** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 46.56 | 36.68 | 30.00 |
| | **T7P7** | $EC_{20}$ and/or $EC_{50}$ | NT | NT | NT |
| | | HE (%) | 26.72 | 17.21 | 23.08 |

*NT* no toxic, as no inhibition in bioluminescence of *V. fischeri* was observed at any tested concentration.

Concerning *F. candida* avoidance tests, all the assays performed fulfilled the validity criteria established by the test guidelines (ISO 2011). The results compiled no statistically significant differences in the dual control assays in the average distribution of organisms between both sides of the test containers (Fisher exact test > 0.05). The null hypothesis assumes an equal distribution of the organisms on both sides of each test container. In most cases, FS properties appear to have no influence on the collembolans avoidance behaviour. For some FS samples, the organisms significantly preferred the test soil in detriment of the OECD soil (Fisher exact test: $p < 0.001$: T1P1, T1P3, T1P4, T2P1, T2P2, T2P3, T2P4), while the majority of the FS soils did not cause a significant avoidance of organisms (Fisher exact test: $p > 0.05$) (Figure III.2A). However, even considering FS samples, one exception was observed in T7P2 sample, where organisms significantly avoided the tested soil, with an average percentage of avoidance of 32% (Fisher exact test: $p < 0.001$) (Figure III.2A). According to the > 80% avoidance criterion for the habitat function proposed by Hund-Rinke et al. (2002), we can assume that FS samples did not compromise habitat quality. Thus, considering the collembolans preference by tested soils and taking into account the low average percentage of avoidance observed in the most sampling points, we could assume that soils in the forest area did not have their habitat function affected, which is in agreement with our expectations. Regarding the results obtained for *F. candida* exposed to agricultural soils, it was shown that organisms significantly avoided several sampling soils (T1P6, T1P7, T2P5, T2P6, T2P7, T3P5, T4P6, T4P7, T5P5, T6P4, T6P5, T6P6, T6P7, T7P4, T7P5, T7P6, T7P7) (Fisher exact test: $p < 0.0001$), in which the avoidance percentage ranged between 37% and 76%, values below the limit of impaired habitat function (80%) (Figure III.2B). However, in some specific samples where less than 30% of the springtails were found, as the particular cases of T1P7, T6P5, T6P6 and T7P7 (Fisher exact test: $p < 0.0001$), the results likely suggested the possible involvement of several compounds into the whole soil matrix that might interfere on organism's sensitivity. As mentioned previously, Lake Vela basin drainage is exposed to nutrients and pesticides as a result of the intense agricultural and livestock activities on the eastern side fields (Abrantes et al. 2006a; Abrantes et al. 2006b).

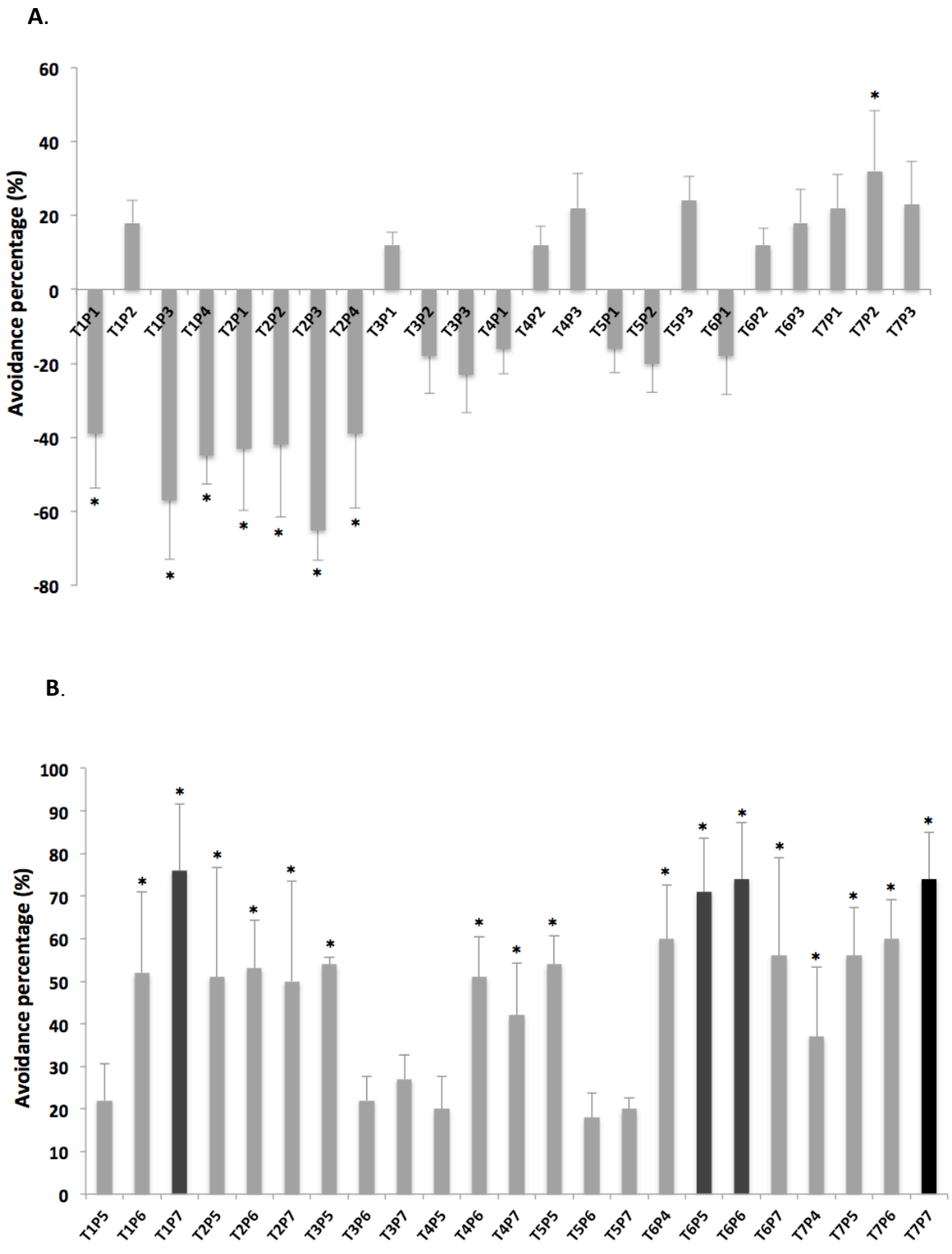


**Figure III.2.** Average percentage of organisms *Folsomia candida* exposed to soils from (**A**) forest (FS) and (**B**) agricultural (AS) fields of Lake Vela, after 48h of exposure to the avoidance test. The bold bars represent that less than 30% of the springtails were found in the samples. The error bars indicate the standard deviation, and asterisks indicate statistical differences ($p \leq 0.05$).

In fact, the natural heterogeneity of soils from both margins of the lake is mostly related with their land-use, and therefore, the local mixture of contaminants and their bioavailability for the terrestrial species could be different. Numerous studies have explored the effects of some pesticide application in agricultural fields to non-target soil organisms, even when used at the recommended field dose. In this context, Santos et al. (2012) reported a significant avoidance of collembolans exposed to chlorpyrifos (64%) and of earthworms exposed to endosulfan (60%), both pesticides applied at recommended field dose. Furthermore, these authors highlighted the impairment of collembolans survival and also the reproductive capacity of both collembolans and earthworms. Another study performed with chlorpyrifos showed significant alterations on *F. candida* instantaneous rate of population increase ($r_i$) and significant reductions in their juvenile production at concentrations of 0.26 µg $g^{-1}$ dry soil and higher (Herbert et al. 2004). Moreover, herbicide Betanal® exerted a significant avoidance on three collembolan species (i.e. *H. nitidus, L. violaceus, O. armatus*) in concentrations higher than 3.5 mg $kg^{-1}$, while *F. candida* avoided the spiked soil at 5.9, 17 mg phenmediphan $kg^{-1}$ soil or higher (Heupel 2002). Although all of these pesticides are not in the list of those most used in this area, available studies demonstrate the sensitivity of collembolans to plant protection products. Another factor that is known to affect the life-cycle parameters of soil-dwelling species is the soil salinity. Regardless soil salinity in temperate climate occurs on a smaller scale (Wichern et al. 2006), organisms might be affected when exposed to natural saline soil. Although soils with salinity values lower than 4000 µS $cm^{-1}$ of electrical conductivity are classified as non-saline (Micheli et al. 2002), the avoidance tests of the earthworms exposed to low salinity (electrical conductivity < 500 µS $cm^{1}$) in both OECD artificial and natural soils, demonstrate that soil organisms are sensitive to lower values of salinity. These authors recorded an avoidance $EC_{50}$ for *Aporrectodea caliginosa* of 260 µS $cm^{-1}$, a value lower than that obtained for *Eisenia fetida* ($EC_{50}$ < 560 µS $cm^{-1}$). In fact, in some sampling points where collembolans significantly avoided AS, the electrical conductivity values recorded were high, in some cases exceeding 400 µS $cm^{-1}$. Moreover, other soil properties can also influence the

behaviour of soil test species in the exposure matrix, as it was already reported by other studies (Römbke et al. 2006; Criel et al. 2008; Natal-da-Luz et al. 2008).

In order to calculate the integrated risk for the EcoToxLoE concerning to the soil habitat function, we combined the results of solid-phase Microtox® test and *F. candida* avoidance responses for each 46 soils. All the samples from FS presented a low risk (IR ≤ 0.50), while AS showed a moderate risk (0.51 ≤ R ≤ 0.75) at five sampling points (T1P7, IR = 0.51; T2P5, IR = 0.75; T6P4, IR = 0.51; T6P5, IR = 0.70; T6P6, IR = 0.61), and high risk (IR > 0.75) specifically at one point (T3P7, IR = 0.85) (Table III.3). A low risk was also observed in the remaining sampling points from AS. In accordance with the results obtained, more specifically the ones where the integrated risk was moderate or high (namely the samples T1P7, T2P5, T3P7, T6P4, T6P5, T6P6), further assays were performed intending to analyse the soil retention function.

**Table III.3.** Calculation of risk values for the ecotoxicological (T1P7, T2P5, T3P7, T5P6, T6P4, T6P5 and T6P6) and chemical (T2P5, T3P7, T5P6 and T6P5) lines of evidence and integrated risk (T2P5, T3P7 and T6P5) for the assessed sampling points.

| | **Sampling points** | | | | | | |
|---|---|---|---|---|---|---|---|
| | **T1P7** | **T2P5** | **T3P7** | **T5P6** | **T6P4** | **T6P5** | **T6P6** |
| **Ecotoxicological** | | | | | | | |
| IR (Soil habitat function) | 0.52 | 0.75 | 0.85 | 0.38 | 0.51 | 0.70 | 0.61 |
| IR (Soil habitat function + + Soil retention function) | 0.39 | **0.60** | **0.71** | n.d | 0.38 | **0.55** | 0.46 |
| **Chemical** | | | | | | | |
| Total metal content | n.d | 0.43 | 0.36 | 0.25 | n.d | 0.42 | n.d |
| **Tier 1 Integrated Risk** | | | | | | | |
| Integrated Risk | n.d | 0.52 | 0.57 | n.d | n.d | 0.49 | n.d |
| Deviation | n.d | 0.21 | 0.43 | n.d | n.d | 0.15 | n.d |

For each sample, values are scaled between 0 and 1 according to Jensen and Mesmen (2006) (see text for details). For the other 39 sampling points, no or low risks for the EcotoxLoE (IR ≤ 0.50) were displayed, and for that reason, IR values were not shown. Bold values highlight samples needing further evaluation.
*n.d* not determined

Therefore, growth inhibition test with algae *R. subcapitata* was performed using the six respective soil elutriates to recalculate the integrated risk for the EcotoxLoE, considering the contribution of both soil habitat function and soil retention function assays (see the following Section 3.3.3).

### 3.3.3. Ecotoxicological assays for the evaluation of soil retention function

All validity criteria were fulfilled in the growth inhibition test with *R. subcapitata*. Generically, the results of the microalgae exposed to soil elutriate revealed less sensitivity compared to those tests where the organisms were exposed to the whole soil matrix. No inhibitory growth effects were recorded for algae at 75 and 100% concentration of elutriates (Figure III.3).

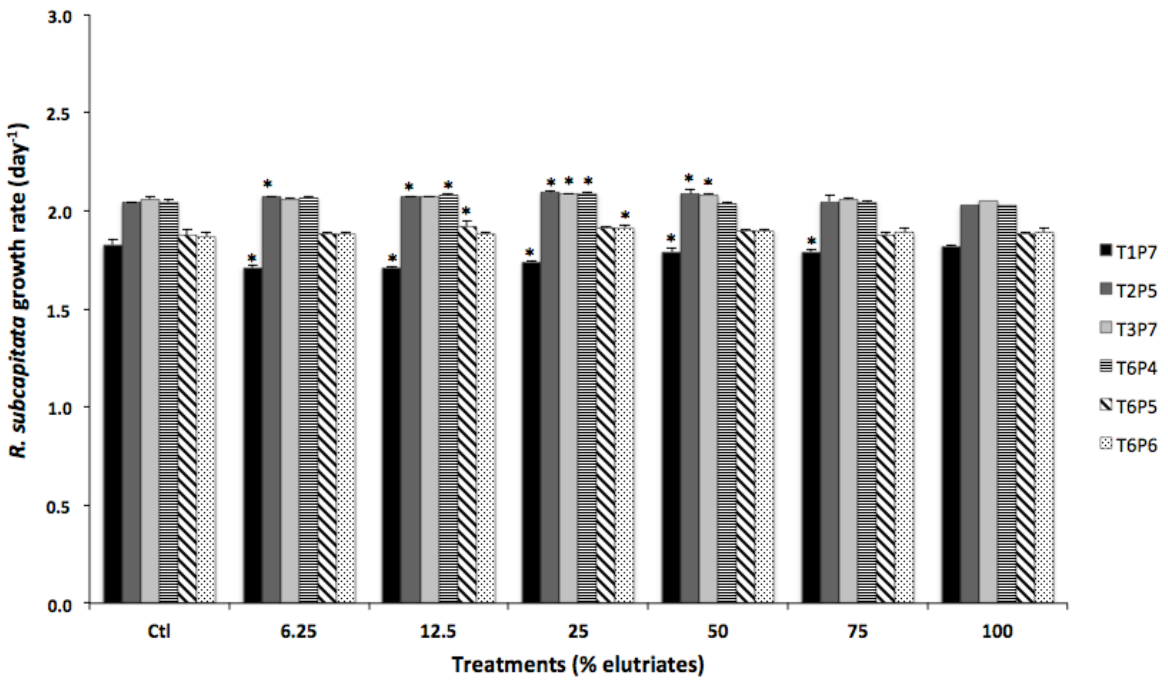


**Figure III.3.** Growth rate ($day^{-1}$) (n=3) of *Raphidocelis subcapitata* exposed to the soil elutriates (T1P7, T2P5, T3P7, T6P4, T6P5 and T6P6) that represented moderate or high risks at soil habitat function evaluation. Error bars represent standard deviation, asterisks assign differences between elutriate dilutions and control (one-way ANOVA followed by Dunnett; $p \leq 0.05$).

In fact, algae exposed to T6P5 did not showed any significant inhibitory effects compared to control (one-way ANOVA: d.f = 6,22; F = 2.557; $p$ = 0.062), while algae exposed to T2P5 (one-way ANOVA: d.f = 6,22; F = 9.345; $p$ < 0.001), T3P7 (one-way ANOVA: d.f = 6,22; F = 12.756; $p$ < 0.001), T6P4 (one-way ANOVA: d.f = 6,22; F = 6.970; $p$ = 0.001) and T6P6 (one-way ANOVA: d.f = 6,22; F = 3.273; $p$ = 0.027) showed a significant stimulation of growth, particularly at low and intermediate concentrations of elutriate (i.e. 6.25, 12.5, 25 and 50%). Only in the case of the elutriate from soil T1P7 (one-way ANOVA: d.f = 6,22; F = 33.774; $p$ < 0.001) a significant inhibitory effect was reported in most dilutions, while no relevant inhibitory effects were observed at the highest elutriate concentration (100%) ($p$ > 0.05). In fact, the stimulation observed in the growth rate of *R. subcapitata* exposed to the soils from the transect 6 (i.e. T6P4, T6P5 and T6P6) was likely due to their proximity to several stables of livestock farming activities and to the explored agricultural fields (Abrantes et al. 2009), which are recognised as a non-point source of nutrients for Lake Vela basin drainage (Abrantes et al. 2006b; Abrantes et al. 2008; Abrantes et al. 2009; Abrantes et al. 2010). In line with our results, Antunes et al. (2010) also observed a consistent stimulation of *R. subcapitata* growth exposed to natural soils spiked with chlorpyrifos and other three commercial formulations of pesticides (at 100% of elutriate test solutions). They concluded that, even in the presence of contaminants, nutrients released from the soils to elutriates were responsible for the observed stimulation of algae growth, masking the toxicity of the pesticides.

Finally, recalculating the integrated risk for the EcotoxLoE by combining the results from soil retention function with those from soil habitat function, we observed that the integrated risk reduced from high to moderate level in T3P7 (0.51 ≤ IR ≤ 0.75), together with a reduction from moderate to low risk (IR ≤ 0.50) in the sampling points T1P7, T6P4 and T6P6 (Table III.3). Despite the slight reduction observed in T2P5 and T6P5, the integrated risk was maintained as moderate. Therefore, for those three sampling points where risk levels remained moderate (T2P5, IR = 0.60; T3P7, IR = 0.71; T6P5, IR = 0.55), we should consider the determination of potential contaminants by the inclusion of the additional chemical data in the risk assessment process. However, for the other 43

sampling points, which include 23 FS and 20 AS samples, presenting no or low risks, we could assume that there are no evidence of risks to soil biota.

### 3.3.4. Pesticide and metal concentrations in soils

Following the approach recommended in the previous section, the soil samples from AS (i.e. T2P5, T3P7 and T6P5), reference soil from FS (i.e. T2P1) and an agricultural soil with no EcotoxLoE risk (i.e. T5P6) were analysed for specific active ingredients of pesticides used by the local farmers in lands near Lake Vela, as well as total metal concentrations. Regarding pesticides, all the substances analysed (i.e. alachlor, aldrin, dieldrin, glyphosate and pendimethalin) were below the detection limit of the analytical apparatus used for their quantification (Supplementary Material: Table SIII.2), and for this reason, pesticide data was not considered to calculate the risk for the ChemLoE. Although some of these compounds can be classified as persistent pollutants (Connell et al. 1999; Zimmerman et al. 2000), their biological degradation (at least to certain level) and losses by percolation or leaching through soil should not be ignored for this area due to the sandy nature of soils from the Lake Vela drainage basin. Furthermore, the applications were also reduced in the most recent years, by legal enforcement that obliges a strict control of application rates of pesticides, as well as the technical training of farmers responsible for such applications.

The total content of metals analysed in the sampling points was all below the $HC_5$ for soil biota and microbial processes previously defined by Jänsch et al. (2007), as it is summarized in Table III.4. The FS-REF sample (T2P1), used as soil reference, presented metal concentrations well below those from all the sampling points analysed, which could be assumed as good reference soil for the calculation of risks in the ChemLoE. Considering the obtained data, the AS samples T2P5, T3P7 and T6P5 presented high concentrations of As, Cd, Cu and Pb compared to T5P6. Moreover, T5P6 presented a similar content of Cr and Ni, and high levels of Zn when compared with the other AS samples analysed. Within these samples, the total content of metals was similar except for Pb content, which was 9 times higher in T3P7 (Table III.4).

**Table III.4.** Total content of metals (mg kg-1 $soil_{dw}$) recorded in AS samples with moderate risk (T2P5, T3P7 and T6P5) and no risk (T5P6) in the ecotoxicological line of evidence and respective FS reference (T2P1) compared to background soil values.

| | As | Cd | Cr | Cu | Ni | Pb | Zn |
|---|---|---|---|---|---|---|---|
| **$HC_5$ Soil Values**[a] | *5.63* | *6.78* | *5.02* | *55* | *64* | *163.5* | *160.3* |
| **FS-REF (T2P1)** | <0.4 | 0.03 | 0.87 | 0.06 | 0.36 | 0.89 | 1.13 |
| **T5P6** | <0.4 | 0.04 | 2.20 | 3.82 | 0.85 | 2.94 | 20.75 |
| **Sampling points** | | | | | | | |
| **T2P5** | 1.74 | 0.12 | 2.85 | 5.54 | 0.81 | 5.41 | 11.06 |
| **T3P7** | <0.4 | 0.11 | 2.08 | 4.03 | 0.77 | 44.73 | 6.30 |
| **T6P5** | 3.37 | 0.03 | 1.40 | 5.07 | 0.84 | 4.36 | 16.17 |

[a] $HC_5$ soil values – hazard concentration for 5% of the species and microbial processes based on $EC_{50}$ values (Jänsch et al. (2007).

The calculation of risk for the ChemLoE revealed that the sampling points T2P5, T3P7 and T6P5, as well as the sample T5P6, had a low risk (IR ≤ 0.50), when the total content of metals was taken into account (Table III.3). However, combining the risk for the EcotoxLoE and the ChemLoE, a slight reduction in the integrated risk was observed for all the samples analysed compared to those values obtained only for the EcotoxLoE evaluation (Table III.3). Furthermore, the sample T5P6, with no ecotoxicological risk, also displayed any risks at the final of the assessment.

### 3.4. Conclusions

In general, the application of ecotoxicological tests for the evaluation of the ecological risk assessment (tier 1) seems to be effective in evaluating the toxicity of soils from Lake Vela basin drainage. Even solid-phase assays are more relevant since they are closest to real situations (Domene et al. 2008), the elutriate assays have a particular interest considering the sandy nature of our soils with a poor organic matter content, factors that likely promote contaminants' mobility for aqueous extracts, as well as its high bioavailability (Maxam et al. 2000). Thus, having into consideration the integrated risk

values of both areas, we could define FS as a reference area, since any environmental risk was detected. By opposite, some AS soils required a more deep analysis. For those limited cases, we performed an additional evaluation of risks based on chemical analysis aiming to complement tier 1 of the ERA with further data that could support cause-effect relationships and also reduce the degree of uncertainty. The chemical data reinforces the conclusions made based on the EcotoxLoE, highlighting the role of soil organisms as more sensitive indicators of the effects of mixtures of soil contaminants. Thus, considering that analytical methods are extremely expensive and that their use could limit the application of the ERA framework, on a routine basis, to areas under diffuse pollution, the approach proposed in our study can provide a feasible evaluation for tier 1, with lower costs. In this study, the cost per sample for pesticides and metal's analysis was about 355 and 15 euros, respectively. Without taking into account the costs of the ecotoxicological assays, if a complete tier 1 was performed, about 17,000 euros were needed to support the costs of the ChemLoE only.

In summary, we consider that our approach can be proposed for extensive areas affected by diffuse pollution. Nevertheless, for reducing even more the uncertainty, the EcotoxLoE should be complemented with more short-term ecotoxicological assays.

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**This article contains Supplementary Material.**

## 3.5. References

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## 3.6. Supplementary material

**Table SIII.1.** Total content of metals (mg kg-1 soildw) recorded in AS samples of Lake Vela that presented moderate risk (T2P5, T3P7 and T6P5) and no risk (T5P6) in the ecotoxicological line of evidence. The sample FS-REF from the forest area (T2P1) served as a reference sample.

| | Ag | Al | Ba | Ca | Co | Fe | K | Mg | Mn | Mo | Na | Sb | Sn | Th | Ti | V | W |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| **FS-REF (T2P1)** | 0.01 | 564.15 | 3.63 | 281.01 | 0.08 | 556.36 | 211.20 | 108.01 | 7.71 | 0.73 | 76.69 | 0.05 | 0.12 | 0.69 | <0.003 | 0.74 | 1.09 |
| **T5P6** | 0.01 | 908.06 | 8.32 | 658.47 | 0.14 | 590.69 | 302.84 | 216.90 | 14.46 | 0.57 | 78.52 | 0.04 | 0.09 | 0.38 | 0.01 | 1.06 | 0.72 |
| **T2P5** | 0.04 | 1594.10 | 11.71 | 2363.93 | 0.17 | 1125.42 | 430.27 | 282.65 | 20.09 | 1.91 | 290.32 | 0.12 | 0.24 | 0.31 | 0.03 | 1.88 | 2.07 |
| **T3P7** | 0.02 | 1168.61 | 10.66 | 633.34 | 0.12 | 816.50 | 346.53 | 280.72 | 8.63 | 0.96 | 80.52 | 0.08 | 0.14 | 0.47 | 0.02 | 1.48 | 1.10 |
| **T6P5** | 0.05 | 1080.88 | 11.61 | 1506.37 | 0.29 | 1967.11 | 233.32 | 165.12 | 66.96 | 2.69 | 239.20 | 0.09 | 0.18 | 0.22 | 0.03 | 1.24 | 2.00 |

**Table SIII.2.** Concentrations (mg $Kg^{-1}$) of pesticides (alachlor, aldrin, dieldrin, glyphosate and pendimethalin) in AS samples collected in the Lake Vela with moderate (T2P5, T3P7, T6P5) and no (T5P6) ecotoxicological risks. The sample FS-REF from forest area (T2P1) was used as a reference sample.

| | Alachlor | Aldrin | Dieldrin | Glyphosate | Pendimethalin |
|---|---|---|---|---|---|
| **FS-REF (T2P1)** | <0.05 | <0.015 | <0.015 | <0.05 | <0.05 |
| **T5P6** | <0.05 | <0.015 | <0.015 | <0.05 | <0.05 |
| **T2P5** | <0.05 | <0.015 | <0.015 | <0.05 | <0.05 |
| **T3P7** | <0.05 | <0.015 | <0.015 | <0.05 | <0.05 |
| **T6P5** | <0.05 | <0.015 | <0.015 | <0.05 | <0.05 |

Limits of detection: Alachlor/Glyphosate/Pendimethalin = 0.05 mg $kg^{-1}$; Aldrin/Dieldrin = 0.015 mg $kg^{-1}$

# Chapter IV

## $TiO_2$ nanoparticles for the remediation of eutrophic shallow freshwater systems: Efficiency and impacts on aquatic biota under a microcosm experiment

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# $TiO_2$ nanoparticles for the remediation of eutrophic shallow freshwater systems: Efficiency and impacts on aquatic biota under a microcosm experiment

**Abstract**

The application of nanomaterials (NMs) in the remediation of eutrophic waters, particularly in the control of internal loading of nutrients, has been started, but limited investigations evaluated the effectiveness of these new treatment approaches and of their potential impacts on species from shallow freshwater lakes. The present work investigated, under a microcosm experiment, the application of a $TiO_2$ nanomaterial both for reducing nutrient (mainly phosphorus and nitrogen forms) desorption and release from sediments (preventive treatment – PT) and for eliminating algal blooms (remediation treatment – RT). Furthermore, we also intended to assess the potential impacts of nano-$TiO_2$ application on key freshwater species.

The results showed the effectiveness of nano-$TiO_2$ in controlling the release of phosphates from surface sediment and the subsequent reduction of total phosphorus in the water column. A reduction in total nitrogen was also observed. Such changes in nutrient dynamics contributed to a progressive inhibition of development of algae after the application of the NM in PT microcosms. Concerning the ability of nano-$TiO_2$ to interact with algal cells, this interaction has likely occurred, mainly in RT, enhancing the formation of aggregates and their rapid settlement, thus reducing the *algal bloom*. Both treatments caused deleterious effects on freshwater species. In PT, *Daphnia magna* and *Lemna minor* showed a significant inhibition of several endpoints. Conversely, no inhibitory effect on the growth of *Chironomus riparius* was recorded. In opposite, *C. riparius* was the most affected species in RT microcosms. Such difference was probably caused by the formation of larger $TiO_2$-algae aggregates in RT, under a high algal density, that rapidly settled in the sediment, becoming less available for pelagic species. In summary, despite the effectiveness of both treatments in controlling internal nutrient loading and in the mitigating algal blooms episodes, their negative effects on biota have to be seriously taken into account.

**Keywords**: Nanomaterials; Microcosm experiments; Remediation; Eutrophic systems; Ecotoxicological impacts

### 4.1. Introduction

Phosphorus (P) is a major nutrient for aquatic ecology, and its excessive supply combined with some other physical or geochemical conditions (Qin et al., 2006) can significantly contribute for the eutrophication of aquatic ecosystems (Nyenje et al., 2010; Wang and Wang, 2009), with impacts on community structure (Arhonditsis and Brett, 2005; Buskey, 2008; Cloern, 2001; Smith et al., 1999) and on the sustainability of ecosystems. Generally, when the external loading of P from catchment sources (e.g. sewage discharges, agricultural wastewaters and diffuse runoffs from agricultural land) increases, sediments of receiving systems start to play a critical role in the overall P dynamics (Fang et al., 2008; Hein, 2006; Hooda et al., 2001; McGechan et al., 2005). Once P enters the water body, sediments have the dual capacity to act either as a sink or as a source releasing the adsorbed P back to the water column. Even when external P loading has been reduced, the internal loading can actually be promoted or maintained in the system, sustaining biological productivity for decades (Wang et al., 2009). This is the case of shallow lakes, where wind-wave forces frequently promote P-cycling between lake sediments and the overlaying water column (Jeppesen et al., 2005; Sondergaard et al., 2001). As an extreme response to the higher loading of nutrients (both from external and internal sources), algal blooms became a widespread occurrence in shallow lake ecosystems (Anderson et al., 2008; Heisler et al., 2008; Li et al., 2014). High biomass blooms commonly cause fish kills via oxygen deficits (Anderson et al., 2008; Glasgow and Burkholder, 1990; Touchette et al., 2007), and when dominated by cyanobacteria, the toxins released can adversely impact aquatic biota (Dai et al., 2008; de Figueiredo et al., 2006; Glasgow and Burkholder, 1990; Vasconcelos et al., 1996) with an overall decrease in biodiversity in the long-term.

To face the eutrophication problem that compromises the overall quality of water, the ecological status and the services provided by freshwater ecosystems, several studies have proposed environmental remediation strategies either for P scavenging or removal from lake sediments. These measurements include physical approaches, such as sediment

dredging (Gil et al., 2010; Yenilmez and Aksoy, 2013), artificial hypolimnetic aeration (Engstrom, 2005; Grochowska and Gawrońska, 2004; Grochowska et al., 2013), as well as geochemical approaches like the application of sediment P capping agents (including Phoslock® commercial formulation) (Lürling and Oosterhout, 2013; Meis et al., 2013, 2012) or the widely used aluminium based salts (Babatunde and Zhao, 2010; Lewandowski et al., 2003; Reitzel et al., 2006). Each approach, used alone or combined, comprises several advantages and disadvantages, which were well compiled by Hickey and Gibbs (2009) and Zamparas and Zacharias (2014). According to these authors, the recognised disadvantages related with some of these recovery techniques are the expensive cost of the products, its impact on downstream waters and sediments, the need for specialized machinery to large-scale applications, and restriction period for drinking water, irrigation and stock watering.

Taking these facts into consideration, a novel approach for the treatment of polluted soils and water has recently emerged. Its application is based on nanostructured materials, including engineered formulations of titanium oxide ($TiO_2$), which have natural attributes that can be explored for environmental remediation (Karn et al., 2009; Sánchez et al., 2011). $TiO_2$ is a naturally occurring mineral that can exist in three crystalline forms, known as rutile, anatase and brookite. However, due to the current wide utilization and the new promising uses, several nanostructured $TiO_2$ nanomaterials (nano-$TiO_2$) have been manufactured since it is expected that nanometer-size (< 100 nm) particles exhibit many special properties compared to the bulk $TiO_2$ material (Adams et al., 2006; Clemente et al., 2013). Specific properties of nano-$TiO_2$, as small particle size, large specific surface area and strong electrostatic attraction at the surface, are known to improve both its photocatalytic activity and its adsorption capacity (Fujishima et al., 2008; Zhang et al., 2007). Titanium oxide (in particular the anatase crystalline form) has been tested, due to their high adsorption capacity (Chekli et al., 2015; Erhayem and Sohn, 2014), for the control of internal loading of nutrients, especially phosphorus. There are evidences that nano-$TiO_2$ may possibly retard the release of sediment phosphorus, and thereby improve P adsorption on sediment (Luo et al., 2010; Zhang et al., 2014), thus mitigating or even preventing eutrophication.

Also, a recent study indicated that $TiO_2$ nanomaterials could successfully be employed on the control of algal growth in eutrophic systems (Hartmann et al., 2010; Kim and Lee, 2005; Ochiai et al., 2010; Rodríguez-González et al., 2010; Wang et al., 2015), mostly due to nano-$TiO_2$ aggregation and sedimentation properties in aqueous suspension ( Hartmann et al., 2010; Campos et al., 2013; Chowdhury et al., 2013), but also due to their photocatalytic potential (Kim et al., 2011; Metzler et al., 2012, 2011). Therefore, it is important to determine how and in what extent the nano-$TiO_2$ application may influence the ecosystem community, particularly the survival and structural endpoints of non-target species of eutrophic systems.

Hence, the objective of this study was to investigate, under a microcosm experiment, whether nano-$TiO_2$ affects the contribution of sediment for internal loading phosphorus, and to what extent chemical properties of nano-$TiO_2$ promote algal aggregation, mitigating algal blooms. Additionally, this study aimed to assess if nano-$TiO_2$ may affect freshwater biota and their populations, when applied for environmental remediation purposes in eutrophic systems. Thus, the macrophyte *Lemna minor* (growth inhibition) and the cladoceran *Daphnia magna* (reproduction and growth endpoints) were used as representative planktonic species and *Chironomus riparius* (growth inhibition) was chosen as a representative benthic species.

## 4.2. Material and Methods

### 4.2.1. Collection of natural samples for microcosm experiments

Sediment and freshwater samples for the microcosm experiments were collected in the beginning of autumn, in a small shallow lake in Portugal (Lake Vela, Figueira da Foz, centre of Portugal). In the last decades, this shallow lake faced serious environmental problems caused by the high input of nutrients from its drainage basin, which was responsible for an advanced and injurious eutrophication process (Abrantes et al., 2009, 2006a, 2006b). The collection point in the shallow lake was located in the east bank, surrounded by agricultural fields, which are contributing to nutrient inputs by surface and sub-surface runoff.

The upper sediment layer (ca. 10 cm depth) was collected with a stainless steel corer into clean and black opaque plastic bags, which were airtightly sealed for safe transport to laboratory at 4$^{o}$C. In the laboratory, sediment samples were sorted out and mixed to get a composite sample, in order to minimize sampling heterogeneity.

Sub-surface water samples were collected and filtered *in situ* through a 60-µm mesh net for 20-L plastic containers. This first filtration step aimed a first elimination of plankton from water. In the laboratory, water samples were again filtered with a 1.2 µm glass microfibre filters (Whatman GFC ∅ 47mm) to remove coarse seston particles. All water and sediment samples were stored in the dark, at 4ºC (maximum storage time: 1 week).

Finally, additional water samples were collected *in situ* to obtain a sample of the phytoplankton community, and immediately transported to the laboratory at 4ºC. At the laboratory, the water samples were centrifuged and algal pellets were separated and kept into refrigerator at 4ºC until further procedure.

### 4.2.2. Phytoplankton inoculum preparation

Phytoplankton was obtained from freshwater samples, collected in Lake Vela (see previous section), after centrifugation (4000 rpm for 15 min). The supernatant was removed by decantation and the pellet was used to start an individual culture in 250 mL Erlenmeyer flasks, with 100 mL of sterilized Woods Hole MBL nutritive culture medium (Stein, 1973). The cultures were maintained under controlled temperature (20 ± 2ºC), photoperiod (16h$^{L}$: 8h$^{D}$) and light intensity (60-120 µE· m$^{-2}$ s$^{-1}$) conditions. To start new cultures, algae were harvested during their exponential growth phase (6-7 days old) and inoculated in a fresh medium.

In order to prepare algal inoculum for the microcosm experiments, four litters of MBL medium were prepared weekly, in glass bottles of 5L, and inoculated with algae from previous cultures. At the exponential growth phase, under the conditions described above, algae were harvested by centrifugation at 4000 rpm during 4 min. The supernatant was discarded and the resulting pellets were resuspended with filtered lake water and applied directly into the microcosms during the experiment.

### 4.2.3. Microcosms experimental design

The description of all steps of the experimental design, the preparation of microcosms and the time schedule are described in Table IV.1. Cylindrical glass jars, 70 cm high, 20 cm of inner diameter and a maximum volume of 22 L were used as microcosms. The external surface of microcosm cylinders was covered with a black opaque paper, placed from the bottom to the height of 30 cm high to prevent lateral light penetration. All the microcosms were filled with a layer of 10 cm of Lake Vela sediment. Afterwards, 50 cm of water (approximately 16 L) were added carefully to avoid sediment resuspension. Each microcosm was gently aerated on the top to promote a depth gradient of dissolved oxygen. The microcosms were placed within a climatic chamber under constant conditions (air temperature: 20 ± 2ºC; light intensity: 6500 Lux; photoperiod: $16h^{L} : 8h^{D}$) and were left undisturbed for one month for sediment-water interface stabilization.

Following the description above, four different treatments were defined, with 4 replicates per treatment (in a total of 16 microcosms): CTL1, sediment + freshwater (filtered through a 1.2 µm filter); CTL2, sediment + freshwater + phytoplankton; PT (Preventive Treatment), sediment + freshwater + phytoplankton + nano-$TiO_2$ (1 g $L^{-1}$) immediately added after the end of stabilization period (day 31) and before the addition of the second algae inoculum; RT (Remediation Treatment), sediment + freshwater + phytoplankton + nano-$TiO_2$ (1 g $L^{-1}$) added (at day 43) when a meaningful phytoplankton growth was recorded (simulating an algal *bloom* occurrence; $OD_{440nm}$ = 0.159 ± 0.020) (Table IV.1). The nano-$TiO_2$ was introduced directly as powder at the surface water of PT and RT microcosms. The concentration of the nanomaterial added was based on our preliminary experiments, in batch conditions, in which a range of concentrations (from 0.25 g $L^{-1}$ to 2 g $L^{-1}$) of nano-$TiO_2$ were tested in order to select the most effective concentration in reducing the algal density. Our results showed that nano-$TiO_2$ at 1 g $L^{-1}$ was the most effective concentration to be applied in the remediation of natural waters (data not shown). To the best of our knowledge, there are no studies reporting the use of titanium oxide to solve eutrophication problems. Only one study from Shephard et al. (1998) reported the use of titanium oxide to remove cyanobacterial microcystin toxins through photocatalytic degradation.

**Table IV.1.** Time schedule, tasks performed and parameters monitored in the microcosms during the stabilization period, the preventive treatment (PT) and the remediation treatment (RT).

| | | Time schedule | Tasks performed | Parameters monitored |
|---|---|---|---|---|
| Stabilization Period | | Day 1 | Microcosm set-up | Nutrients; Physico-chemical parameters; $OD_{440nm}$ |
| | | Day 2 | First addition of algae inoculum in CTL2, PT and RT | |
| | | Day 10 | | Nutrients; Physico-chemical parameters; $OD_{440nm}$ |
| | | Day 20 | | Nutrients; Physico-chemical parameters; $OD_{440nm}$ |
| | | Day 30 | End of stabilization period | Nutrients; Physico-chemical parameters;$OD_{440nm}$ |
| Preventive Treatment (PT) | | Day 31 | Nano-$TiO_2$ added to PT replicates | |
| | | Day 32 | | Nutrients (PT*); $OD_{440nm}$ |
| | | Day 33 | | Nutrients (PT*); $OD_{440nm}$ |
| | | Day 34 | | Nutrients (PT*); $OD_{440nm}$ |
| | | Day 35 | | Nutrients (PT*) |
| | | Day 36 | Second adittion of algae inoculum in CTL2, PT and RT | Nutrients (PT*); Physico-chemical parameters; $OD_{440nm}$ |
| | | Day 40 | | Nutrients (PT*) |
| | Remediation Treatment (RT) | Day 43 | Nano-TiO2 added to RT replicates | Nutrients; $OD_{440nm}$** |
| | | | Beggining of *D.magna* chronic bioassays | Physico-chemical parameters** |
| | | | Beginninng of *L. minor* growth inhibition tests | |
| | | Day 44 | | Nutrients (RT*); $OD_{440nm}$ |
| | | Day 45 | | Nutrients (RT*); $OD_{440nm}$ |
| | | Day 46 | | Nutrients (RT*); $OD_{440nm}$ |
| | | Day 47 | | Nutrients (RT*); $OD_{440nm}$ |
| | | Day 48 | | Nutrients; $OD_{440nm}$ |
| | | Day 50 | End of 7-d *L. minor* growth inhibition test | |
| | | Day 53 | | Physico-chemical parameters |
| | | Day 55 | | Nutrients (RT*); $OD_{440nm}$ |
| | | Day 60 | | Nutrients (RT*); $OD_{440nm}$ |
| | | Day 64 | End of *D. magna* chronic bioassays | Nutrients; Physico-chemical parameters; $OD_{440nm}$ |
| | | | End of 21-d *L. minor* growth inhibition tests | |
| | | Day 65 | Beggining of *C. riparius* growth inhibition tests | |
| | | Day 75 | End of *C. riparius* growth inhibition tests | |
| | | | End of the microcosm experiment | |

PT - Preventive treatment; RT – Remediation treatment; CTL1 – Control without phytoplankton; CTL2 – Control with phytoplankton.
PT*- parameter only measured in the PT replicates; RT*- parameter only measured in the RT replicates; **-parameter evaluated before the addition of $TiO_2$

These authors concluded that to be effective in natural waters concentrations up to 5 g $L^{-1}$ of the catalyst have to be used. However, only the photocatalytic effect was taken into consideration. Furthermore, it was reported that nano-$TiO_2$ did not cause significant environmental impacts on freshwater algae at concentrations up to 300 mg $L^{-1}$ (Kulacki et al., 2012).

The replicates of control CTL2 and of PT and RT treatments were inoculated with phytoplankton collected in the Lake Vela (see Section 4.2.2). Thus, 15 mL of inoculum (at dilution ratio 1:20; absorbance value at 440 nm = 0.354 ± 0.002) were added once to each microcosm at the beginning of the stabilization period (at day 2) (Table IV.1). Moreover, at day 36, a new addition of the same inoculum (Table IV.1), in identical volume, was performed to CTL2, PT and RT, in order to simulate an *algal bloom* in RT and also to evaluate the phytoplankton dynamics in PT.

The experiment followed a stepwise approach with three distinct periods: the stabilization period that lasted 30 days; the preventive treatment period extended between days 31 and 75, and the remediation period which begun at day 43 and finished at day 75. Hence, the PT and RT treatments started at different moments, but occurred in parallel from day 43 until the last day of the experiment (day 75) (Table IV.1).

Titanium (IV) oxide nanopowder (particle size < 25 nm, 99.7% trace metal basis), purchased from Sigma-Aldrich Corporation, was used in the experiments. According to the manufacturer, the crystal phase of nano-$TiO_2$ was 100% anatase, and the specific surface area was 45-55 $m^2/g$ with a density of 3.9 g m $L^{-1}$.

### 4.2.4. Physical and chemical analyses

During the stabilization period, water samples of 60 mL were collected from each microcosm at days 1, 10, 20 and 30 to follow changes in the concentration of total phosphorus, total nitrogen, phosphates and nitrates. At the days 10, 20 and 30 of the stabilization period, the level of the water in each microcosm was reset with filtered (through a 1.2 µm filter) water from the lake. Although a minimum volume was needed to replace water lost by evaporation, the main objective was to prevent further and more meaningful replenishments during the treatments. After the addition of nano-$TiO_2$ into PT

and RT microcosm treatments, all nutrients were monitored at each 24h, during 5 consecutive days. Subsequent measurements were made frequently, as shown in Table IV.1. In day 64 such measurements were finished, because it was expected that the presence of larval food in the water would interfere with nutrients dynamics (see Section 4.2.6).

Nutrients were analysed according to APHA (1998) methodologies. The phosphates and nitrates were analysed in filtered water (through a 1.2 µm filter), while total phosphorus and total nitrogen were analysed in non-filtered water. Total phosphorus was determined after mineralization of water samples by the acidic persulfate method followed by the ascorbic acid procedure. Total nitrogen was assayed by oxidation with alkaline persulfate to nitrate. Nitrates were determined by the cadmium reduction method and phosphates (in the form of orthophosphates – $PO_4^{3-}$) were determined by the ascorbic acid procedure.

Parameters like water temperature, pH, conductivity and concentration of dissolved oxygen of each microcosm were measured (WTW MULTI 3430) until day 64. Additionally, changes in phytoplankton biomass/density were indirectly followed through the measurement of optical density at 440 nm in a UV-VIS spectrophotometer - $OD_{440nm}$).

### 4.2.5. Test species and culture conditions

Cladocerans from the species *Daphnia magna* were obtained from a laboratory stock culture and maintained in synthetic ASTM hard water medium (ASTM, 1996) supplied with an organic additive extract composed by the algae *Ascophyllum nodosum* (Baird et al., 1989) at constant temperature (20 ± 2ºC), photoperiod ($16h^L$:$8h^D$), and light intensity (not exceeding 15-20 $\mu E \cdot m^{-2} \cdot s^{-1}$). *D. magna* culture medium was renewed three times a week and the organisms were fed with *R. subcapitata* at rate of 3.0 X $10^5$ cells/mL/day. Less than 24 h old neonates from the third to the fifth broods were used for microcosm experiments.

The macrophyta *Lemna minor* was collected in a pond and maintained in the laboratory as a successful long-term culture in Steinberg culture medium (OECD, 2006).

Cultures of *L. minor* were renewed once a week and maintained at a controlled temperature (20 ± 2ºC), photoperiod ($16h^{L}$:$8h^{D}$) and light intensity (about 6500 Lux).

*Chironomus riparius* larvae were obtained from laboratorial cultures maintained in plastic beakers with a 2 cm layer of commercial sand (< 1mm) previously acid-washed and burned, and reconstituted ASTM hard water (ASTM, 2002; OECD, 2004).

Each beaker was supplied with slight aeration. The culture was maintained in standard conditions, at 20 ± 2ºC and $16h^{L}$:$8h^{D}$ photoperiod. Freshly laid egg masses were transferred from the plastic beakers to glass crystallizing dishes with culture medium until hatching, and the first instar larvae (2-3 days post-hatch) were used to start a new culture of the microcosm experiments. Water and sediment in the culture were renewed every week and the larvae fed (1 mg $animal^{-1}$ $day^{-1}$) with a suspension of ground TetraMin® (Tetrawerke, Germany) twice a week.

### 4.2.6. Microcosm ecotoxicity experiments

In order to test the chronic effects caused by the addition of nano-$TiO_2$, both to prevent algal blooms (PT) and to eliminate them (RT), chronic exposures with the cladoceran *Daphnia magna*, the aquatic plant *Lemna minor* and the benthic invertebrate *Chironomus riparius* were carried out in the microcosms (Table IV.1). For this purpose, the test chambers used for *D. magna* were adapted from those used and validated by Pereira et al. (1999). Chambers (60-mL polypropylene flasks with a lid) were designed for direct contact with microcosm water and hence two lateral squared windows (2 cm side length) were opened and covered with 50µm nylon mesh. The net used allowed the continuous flow of the water through the chamber. One neonate (< 24-h old; from the third brood) was randomly assigned to each chamber and four replicates, fixed with a plastic wire, were established per microcosm for CTL2, PT and RT treatments. All these treatments were supplied with phytoplankton from the lake, which served as food for daphnids. In the control CTL2, organisms were exposed to conditions similar to those found in the Lake Vela, while organisms from treatments PT and RT were exposed to the same conditions, but with nano-$TiO_2$ added at two different situations/moments (Table IV.1).

During the experiment, daphnids were kept under the conditions already described for microcosm maintenance. Dissolved oxygen, pH, temperature and conductivity were monitored throughout the exposure for validation purposes. Every 24h the chambers were retrieved and checked for survival and later for reproduction. The neonates released from the brood pouch were counted and removed from the chambers. The body length of parent females was also determined by extrapolation from moult exopodite length at the beginning of the test and by measuring the exopodite length of daphnids at the end of the test, allowing the calculation of the somatic growth rate. According to the OECD guideline 211 (OECD, 2012), the chronic exposure of daphnids lasted 21 days.

The growth inhibition test with *L. minor* followed the OECD guideline 221 (OECD, 2006), with adaptations, and occurred simultaneously with the chronic exposure of daphnids. The test conditions for *L. minor* were the same as described for the microcosms. Eight colonies with four fronds each were harvested from the laboratory culture and randomly assigned into the top of each microcosm (CTL2, PT and RT). Ten additional groups composed by eight colonies with four fronds each were collected from the culture and dried at 60ºC overnight to provide the initial dry weight for later growth-related calculations. Changes in the growth of *L. minor* after 7 and 21 days of exposure were assessed based on the number of fronds produced (Table IV.1).

The exposure of *C. riparius* started after *D. magna* and *L. minor* had been retrieved from the microcosms, because the presence of food for larvae in the water may interfere with nutrient dynamics and with exposures to nano-$TiO_2$ of both species. Hence, in the beginning of the exposure of *C. riparius* (day 65, Table IV.1) ten first instar larvae were added to each microcosm of CTL2, PT and RT treatments, with a plastic pipette. While adding the chironomids, aeration was stopped for a 30-min period, allowing larvae to settle properly. The food (ground TetraMin® fish food) was added at the top of each column, every other day (in a single dose of 1 mg $animal^{-1}$ $day^{-1}$) to ensure that *C. riparius* larvae had the necessary food for an optimal growth and development.

Assuming that the addition of food used to feed *C. riparius* larvae may alter the water quality and therefore the microcosms balance, the measurement of optical density and nutrient levels ended at day 64, just before the exposure of *C. riparius* (day 65) (Table

IV.1). Only oxygen and pH levels were monitored for assays validation purpose (data not shown). According to the OECD guideline 218 (OECD, 2004), body length of ten larvae from the same group was measured on the beginning of the test to determine average initial body length.

At the end of the experiment (day 75), water was discarded and sediment was taken out of each microcosm. Test organisms were manually collected. The surviving larvae were counted and killed with an acetone:water (1:3) mixture, and body length measurements of larvae were made under a binocular stereoscope. The average final body length (mm) of larvae per vessel is presented.

#### 4.2.7. Data analyses

With regards to nutrient concentrations, a two-way analysis of variance (ANOVA) was performed to test for the significant effect of the treatment and the time at which these parameters were measured throughout the experiment. For the phytoplankton optical density ($OD_{440nm}$) the same statistical analysis approach was followed. When significant interactions ($p < 0.05$) between both factors were observed, differences among treatments were tested using one-way ANOVAs, followed by a Tukey test, with correction for simple main effects (Quinn and Keough, 2002).

For the growth inhibition test with *L. minor* and *C. riparius*, and for the chronic assay with *D. magna*, one-way ANOVAs followed by Tukey tests were employed to find out potential significant differences in the endpoints assessed between the tested treatments, using the software SPSS version 21.0 (SPSS Corporation, Chicago, IL).

### 4.3. Results

#### 4.3.1. Stabilization period (from day 1 to day 30)

Throughout the stabilization period, the level of dissolved oxygen at the surface of the water column remained practically unchanged (Supplementary material, Table SIV.1), but an oxygen gradient was formed inside the microcosms, with lower values recorded near the sediment (ranging from 1.12 to 2.58 mg $L^{-1}$ in all the treatments). The same tendency was recorded for the pH levels, while conductivity values showed a slight increase both in

the controls (CTL1 and CTL2) and in treatments (PT and RT) (Figure IV.1). Regarding nutrients, a progressive but slight increase in total P in CTL2 was observed during the stabilization period. Total P and N were not measured in CTL1, since, as previously explained, water was filtered to remove coarse seston particles in order to assess the contribution of sediment in releasing nutrients (dissolved forms) to the water column and to better perceive changes in $OD_{440nm}$, which was taken to be an indirect measurement of changes in phytoplankton density. Total P content in PT and RT microcosms presented also a similar but gentle increase along the stabilization period and no significant differences were recorded between treatments for this parameter at the end of the stabilization period (Supplementary material, Table SIV.2). In accordance with this trend, a parallel increase of orthophosphates content in all treatments along the stabilization period was observed, but this time also in CTL1 (Figure IV.1). Total N significantly decreased during this period and nitrates showed slight variations in their content between days, but no significant differences were recorded between treatments at the end of this period for either parameter; the interaction between time/treatment was also not significant. Regarding $OD_{440nm}$ values, there were some variations along the stabilization period, especially between CTL1 (without phytoplankton inoculation) and both PT and RT treatments (Figure IV.1). However, no differences were recorded between microcosms to which the phytoplankton was added (CTL2, PT and RT), thus meaning that phytoplankton had a similar but slow development in these treatments. In general, the microcosms from all treatments were considered stable after the 30-day stabilization period, regarding nutrient levels and $OD_{440nm}$, considering that no significant differences were recorded between treatments and the interaction between time/treatments was also not significant (Supplementary material, Table SIV.2).

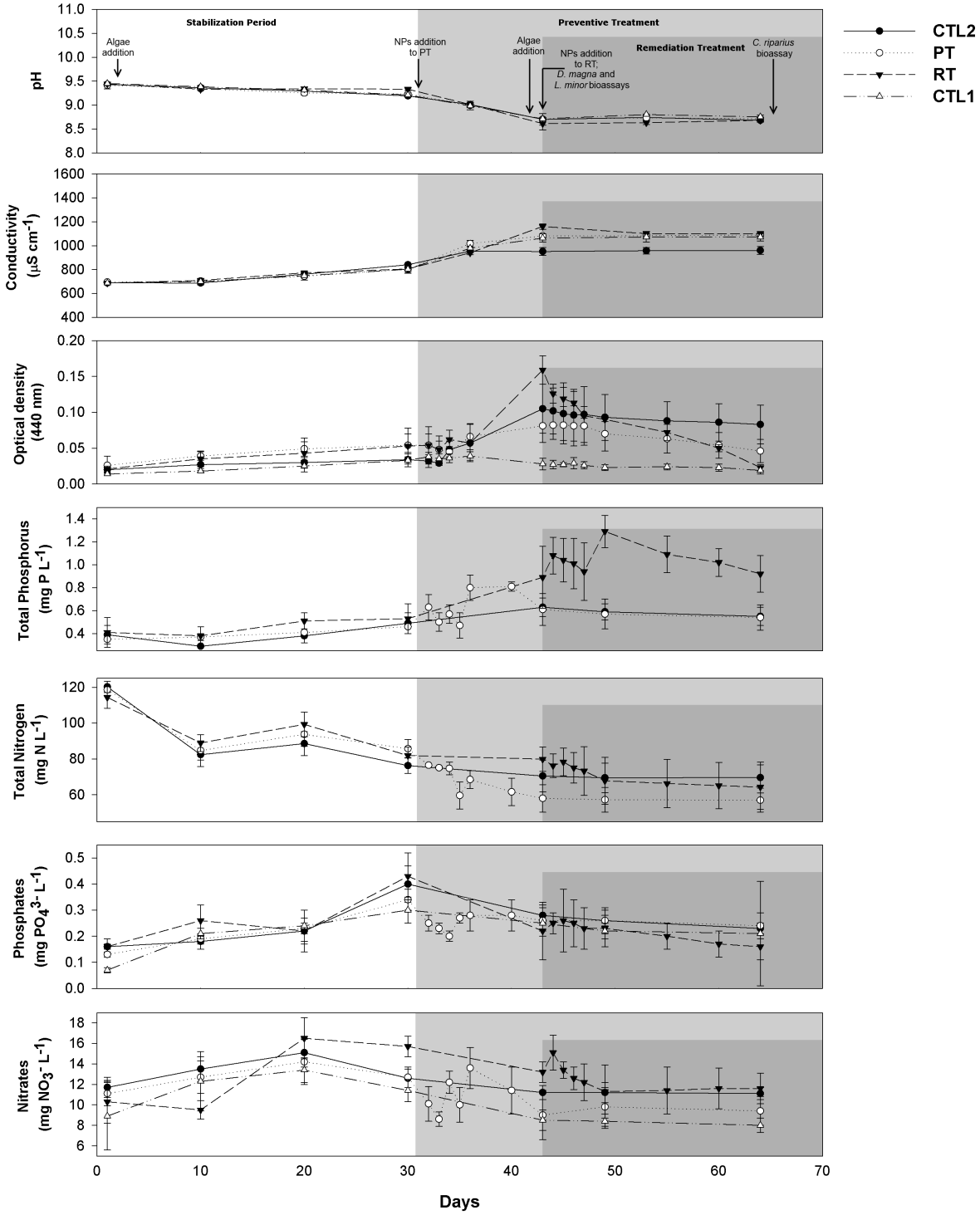


**Figure IV.1.** Variation of physical and chemical parameters and of nutrients measured in the water column of the microcosms, during the different treatment periods of the experiment. The values presented are the means ± standard deviation.

### 4.3.2. Preventive treatment (from day 31 to day 64)

Throughout the days of the PT period, the following variation in the physical and chemical parameters was recorded in the different microcosms: dissolved oxygen levels remained practically unchanged (Supplementary material, Table SIV.1), while conductivity generically increased in all the treatments (Figure IV.1) and such increments seemed to be not related with the addition of the nanomaterial. As far as the pH is considered, the values decreased between days 33-43, however no remarkable differences were found between treatments (CTL1, CTL2, PT and RT). As far as nutrients are considered, in the days after the application of the nanomaterial in PT microcosms, there were some variations in total P content, but this period was characterized by a generic and significant total P increase between day 32 (0.63 mg $L^{-1}$) and day 40 (0.81 mg $L^{-1}$) (Figure IV.1). Nevertheless, particularly from day 43 (after the second inoculation with algae) until the end of nutrient monitoring (day 64), the total P values recorded in PT treatment decreased to values similar to those observed in the CTL2. For the same period, a significant decrease of total N contents throughout the days was observed in the PT treatment. However, despite some variation during the PT period, no significant alterations were observed on dissolved orthophosphate and nitrate levels (Figure IV.1), either between days and treatments (Supplementary material, Table SIV.2). Concerning the $OD_{440nm}$, a slight decrease occurred in the PT treatments immediately after nano-$TiO_2$ addition (day 31) (Figure IV.1). However, and despite a second inoculation of algae in PT microcosms, at day 36 (Table IV.1), which caused an increase in $OD_{440nm}$ levels, this parameter showed a progressive decrease until day 64, to values similar to those recorded in CTL1 (without phytoplankton).

### 4.3.3. Remediation treatment (from day 43 to day 64)

In order to start the RT experiment, the microcosms were inoculated again with algae from the lake at day 36 (Table IV.1). The algae were left to growth and $OD_{440nm}$ values showed a remarkable increase in CTL2, PT and RT until day 43 ($OD_{440nm}$ maximum value of 0.159) in comparison with CTL1 with values lower than 0.030 (Figure IV.1). As soon as evidences of an *algal bloom* occurrence in RT microcosms were obtained, nano-$TiO_2$ was

immediately added to RT replicates aimed in remediating this environmental episode (Table IV.1). No meaningful changes in pH and in dissolved oxygen levels were recorded during this period in RT microcosms (Supplementary material, Table SIV.1). A slight increase in conductivity was recorded on day 43, but it was no caused by the nanomaterial since this parameter was measured before its addition. However, in the next few days, conductivity values stabilised for levels similar to those recorded on CTL1 and on PT (Figure IV.1). Concerning total P content, remarkable variations were recorded, particularly a significant increase immediately after nano-$TiO_2$ addition (maximum value observed at day 49, 1.29 mg $L^{-1}$) (Figure IV.1). Thus, significant differences were recorded along time and also between treatments (Supplementary material, Table SIV.2). The levels of total P in RT microcosms, although displaying a tendency towards reduction (after day 55), were always higher than those recorded in CTL2 and PT, until day 64. Total N also displayed significant differences between days and a significant time/treatment interaction (Supplementary material, Table SIV.2). In fact, despite the decreasing tendency, total P and N contents were constantly increased at RT microcosms during this period. Nitrates content only presented differences between treatments (Figure IV.1) with higher values in the CTL2 and RT treatments during this period (day 44-64), while orthophosphates only presented significant differences between RT and PT (at day 64), being higher at PT. As far as the $OD_{440nm}$ is considered, the highest levels observed at day 43, after the second inoculation of algae, suffered a progressive decrease until day 64 caused by the addition of nano-$TiO_2$ to the RT microcosms (Figure IV.1). This abrupt variation caused significant differences between treatments as well between days for this parameter. A similar decreasing trend was observed in PT microcosms, however less abrupt during this period. At the end of this period, $OD_{440nm}$ values in RT microcosms attained average levels similar to those recorded in the CTL1 (without phytoplankton), while slightly, but non-significantly, higher values were observed in PT microcosms at the end of the experiment.

### 4.3.4. Ecotoxicological evaluation of treatments

Regarding bioassays, all the tests performed (among days 43 and 75) fulfilled the validity criteria required by respective guidelines (OECD, 2012, 2006, 2004). The results obtained in the growth inhibition test with *D. magna* showed significant differences among treatments (one-way ANOVA: d.f. = 2,9; F = 6.052; $p$ = 0.022). In fact, the organisms exposed to the PT treatment showed significant inhibitory effects, only when compared to the CTL2 (Tukey test: $p$ = 0.017; Figure IV.2A). Considering the number of neonates produced, both nano-$TiO_2$ treatments affected this endpoint. The number of neonates born was significantly reduced, and declined 69% in PT and 35% in RT compared to CTL2 (one way ANOVA: d.f. = 2,9; F = 90.306; $p$ < 0.001) (Figure IV.2B). Regarding the number of broods, no statistically significant differences were recorded between treatments (one-way ANOVA: d.f = 2,9; F = 1.900; $p$ = 0.205) (Figure IV.2C). Finally, the intrinsic population growth rate ($r$) was also significantly reduced in both PT and RT microcosms when compared to CTL2 (one-way ANOVA: d.f. = 2,33; F = 150.368; $p$ < 0.001; Tukey test: $p$ < 0.001) (Figure IV.2D).

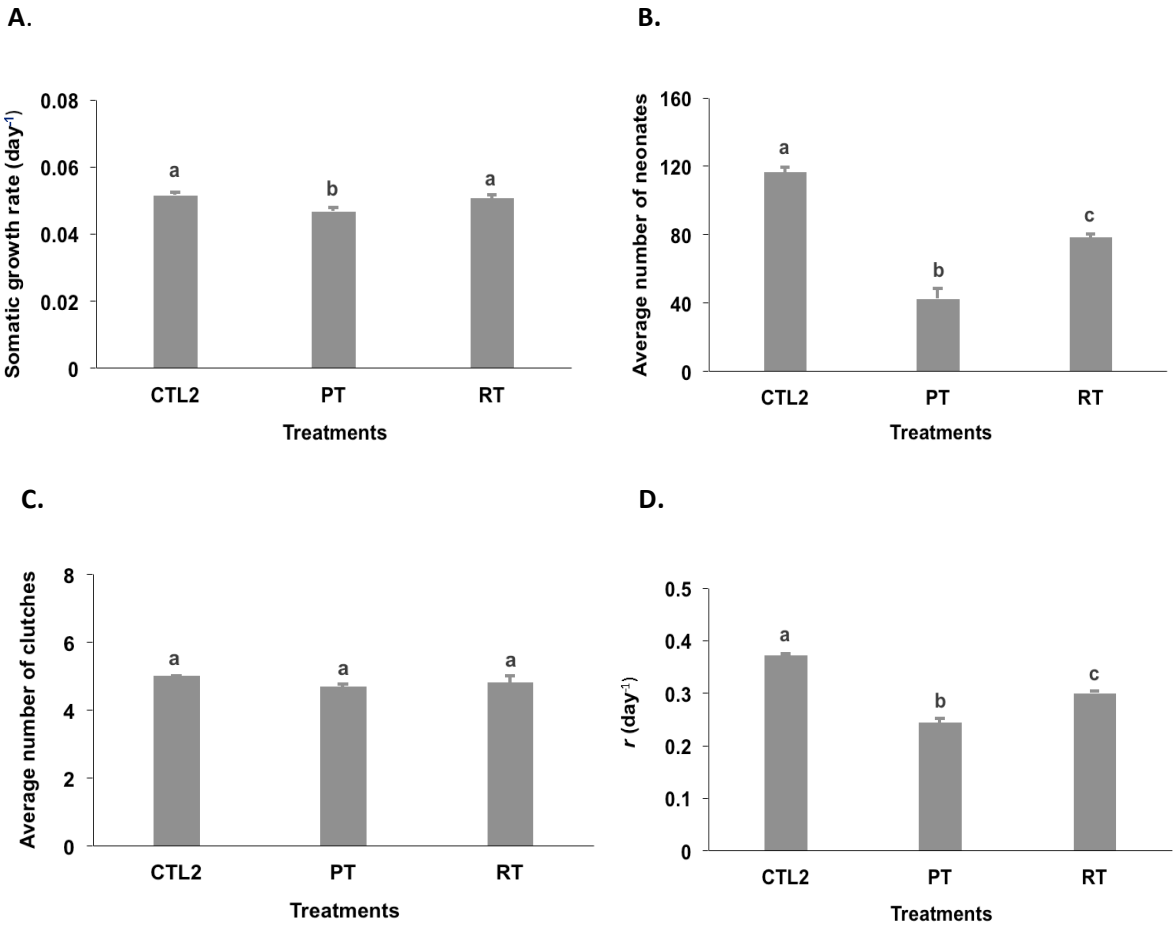


**Figure IV.2**. Average somatic growth rate (**A**), average number of neonates (**B**), average number of clutches (**C**) and growth of population increase (*r*) (**D**) of *D. magna* exposed to control (CTL2), preventive (PT) and remediation (RT) treatments during 21-days chronic test. Error bars represent the standard error. Bars labeled with the same letters are not significantly different (Tukey's HSD, $p < 0.05$ following ANOVA).

After 7 days of exposure, significant differences in the number of fronds of *L. minor* were recorded among treatments (one-way ANOVA: d.f = 2,9; F = 34.112; $p < 0.001$; Figure IV.3). This parameter was significantly reduced in the PT when compared to CTL2 and RT (Tukey test: $p < 0.05$). Fronds from PT became colourless, compared to plants from CTL2 and RT. After 21 days of exposure, the same trend was observed (d.f = 2,9; F = 10.949; $p = 0.004$).

Again, this endpoint was reduced more at PT microcosms when compared to RT and CTL2 (Tukey test: $p < 0.05$).

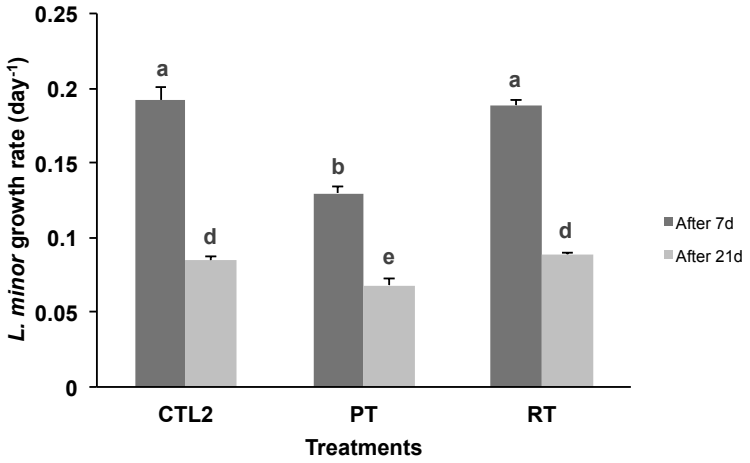


**Figure IV.3.** Average growth rate ($day^{-1}$) of *L. minor* exposed to the control (CTL2), preventive (PT) and remediation (RT) treatments based on the number of fronds after 7 and 21 days. Error bars represent the standard error. Bars labelled with the same letters are not significantly different (Tukey's HSD, $p < 0.05$ following ANOVA).

*C. riparius* growth, determined based on the body length of organisms, showed significant differences among treatments (d.f = 2,11; F = 32.891; $p < 0.001$). The results displayed a significant decrease of this endpoint in RT when compared to CTL2 and PT (Tukey test: $p < 0.001$; Figure IV.4). Conversely, no significant differences were detected between CTL2 and PT (Tukey test: $p = 0.41$).

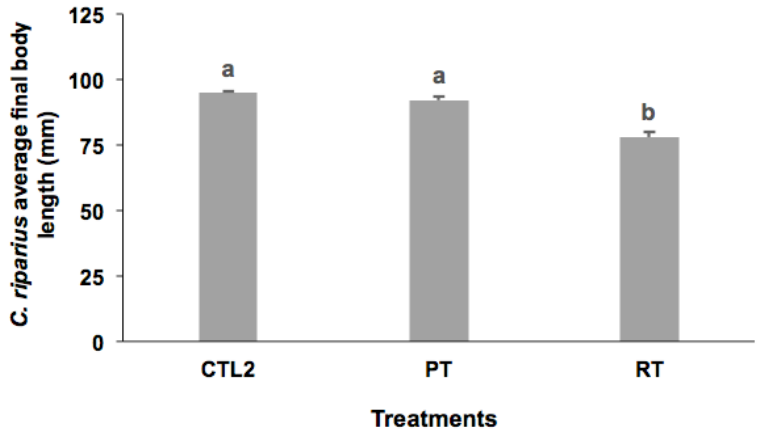


**Figure IV.4**. Average final body length (mm) of *C. riparius* larvae, following a 10-day exposure period to control (CTL2), preventive (PT) and remediation (RT) treatments, based on body length of the larvae. Error bars represent the standard error. Bars labeled with the same letters are not significantly different (Tukey's HSD, $p < 0.05$ following ANOVA).

## 4.4. Discussion

### 4.4.1. Microcosms stabilization period

Concerning the stabilization period, we hypothesized the contribution of the lake sediment in releasing phosphorus to the water column, as soon as an oxygen gradient was established within the microcosms. According to several studies, one of the most important factors affecting the P concentration in water bodies is their release from sediments, a process controlled by some physical and chemical parameters, such as temperature, pH and anaerobic conditions (Ahlgren et al., 2011; Dittrich et al., 2011; Perkins and Underwood, 2001). The pH has been extensively studied for its critical impact on nutrients bioavailability, especially in the interface of overlying water and sediments. It was suggested that at high pH levels (from 8 to 12) P can be released from ferric complexes, due to the competition between hydroxyl ions and the bound P ions (Kim et al., 2003; Liu et al., 2013). The pH values observed during the stabilization period in the microcosms were high, ranging from 9.19 ± 0.01 to 9.45 ± 0.03, and such pH values might have contributed to the release of dissolved phosphorus forms from the lake sediment, along with temperatures varying from 20.2ºC to 20.6ºC. Besides these environmental parameters, the amount of sediment-bound contaminants and nutrients also depends on the level of dissolved oxygen in the overlying water. Several studies observed that phosphorus release, under anaerobic environment, was much greater than in an aerobic environment, which may stimulate the growth of algae and promote the *algal bloom* in overlying water (Gomez et al., 1999; Jiang et al., 2008). In this study, considering the oxygen gradient obtained in all microcosms, particularly the low oxygen values recorded close to the sediment (minimum value, 1.12 ± 0.03 mg $L^{-1}$), the increase of orthophosphate level observed during the stabilization period may have resulted from the phosphate release from sediments. Further, the increase of orthophosphate level in the water column favoured the phytoplankton development observed in some treatments (especially PT and RT) until day 30, as it demonstrated by a slight but non-significant increase of total P and $OD_{440nm}$ values.

On the other hand, total N decreased during this period, suggesting that different factors and processes may have been involved in its balance in the microcosms as

compared to phosphorus. As far as denitrification is considered, it is a process that primarily takes place in the presence of low oxygen levels at the sediment-water interface (Cottingham et al., 1999; Grochowska and Gawrońska, 2004; Jeppesen et al., 1998), which could also be enhanced by high temperatures (Seitzinger, 1988; Zhang et al., 2014). According Seitzinger (1988), different sediments display different denitrification ratios. This process is responsible for removing nitrogen from the system, which in parallel with P release from the sediment may have reduced the ratio TN/TP, limiting the growth of algae. In fact, as demonstrated by the $OD_{440nm}$ recorded, only a slight but non-significant increase in phytoplankton development was observed in the microcosms during this period. Some authors have also demonstrated that pH values higher than 8 may inhibit denitrification (Gao et al., 2012; Zhang et al., 2014), while others argue that the optimal pH is site-specific due to the acclimation and adaptation of the microbial community (Rivett et al., 2008). In fact, the values of pH recorded in the microcosm during the 64 days (ranging between 8.61 ± 0.13 and 9.45 ± 0.03) were high (although with some variation) but within the values reported for Lake Vela (Abrantes et al., 2006a; Castro et al., 2005). Zhang et al., (2014) also proposed that under alkaline conditions the ammonium released from sediments (due to nitrification inhibition) could react with $OH^-$ in the overlying water being converted directly to $NH_3$, and lost from the water. The release of orthophosphates from sediments in the microcosms contributed to levels in the overlying water that were well above the threshold of 100 µg $L^{-1}$ characteristic of hyper-eutrophic systems (Wetzel, 2001). After 30 days, the microcosms were considered stable, as no significant differences were recorded between treatments for any of the parameters measured.

### 4.4.2. Preventive treatment efficiency and ecotoxicological impacts on biota

In opposite to expectations, total P content in the preventive treatment increased after nano-$TiO_2$ addition (maximum value observed at day 40 was 0.81 ± 0.04 mg $L^{-1}$), probably due to the slight phytoplankton increase (at day 36), since total P content includes phosphorus incorporated into phytoplankton biomass (Jeppesen et al., 2005; Reitzel et al., 2006). However, from day 40 until the end of the experiment (day 64), a continuous

decrease of total P content was observed in PT to values similar to those from CTL2. This was likely caused by the progressive inhibition of phytoplankton development as shown by $OD_{440nm}$ values, despite the second inoculation of microcosms with algae on day 36. Nevertheless, $OD_{440nm}$ values at PT were always higher than those recorded on CTL1, indicating that some biological productivity was maintained in PT microcosms until the day 64. Considering $PO_4^{3-}$, which is the form of P that is most bioavailable and readily removed by physical-chemical treatment processes (Boyer et al. 2011), nano-$TiO_2$ seemed to be effective in reducing phosphate concentration in PT, as it was expressed by the water analyses. Considering that the phosphate increase during the stabilization period was promoted by release from sediment fractions (values varied from 0.13 ± 0.01 to 0.34 ± 0.04 mg $L^{-1}$), an effective reduction was recorded after nano-$TiO_2$ application in PT at day 31 (to values close to 0.20 mg $L^{-1}$). The $PO_4^{3-}$ concentration increased again for some days, but then was kept low until the end of the experiment (value at day 64, 0.24 ± 0.05 mg $L^{-1}$). In fact, the control of $PO_4^{3-}$ released from sediment may have contributed for reducing total P concentration from day 43 to the end of the experiment. However, as previously mentioned, some biological productivity was maintained. Similar to our approach, Boyer et al. (2011) used a hybrid anion exchange resin impregnated with iron oxide nanoparticles (PSR, phosphate selective resin) to remove total P (TP) and phosphates ($PO_4^{3-}$) from surface water in an eutrophic lake. These authors showed effectiveness of PRS in removing phosphates (greater efficiency in removing $PO_4^{3-}$ than TP) because of selective inner-sphere complex between $PO_4^{3-}$ and ferric oxide in PSR, with minimal undesirable secondary changes in water chemistry. Also, in the context of eutrophic water remediation, Oliveira et al. (2012) recorded that hybrid nanocomposites containing aluminium nanoparticles (HPNs) can easily adsorb orthophosphates (HNPs removal capacity varied from 0.80 ± 0.01 to 1.27 ± 0.02 mg $g^{-1}$), due to $PO_4^{3-}$ small size and less complexity compared to other anionic forms. Furthermore, Yuan and Wu (2007) reported a large capacity of an nanoclay allophane for adsorbing and precipitating phosphates in aqueous solutions (phosphate concentration decreased from 0.96 to 0.55 mg $L^{-1}$, in 24h of contact), and for that reason they considered this natural nanoparticle as highly promising in the remediation of eutrophic waters. Regarding $TiO_2$ in particular,

Connor and McQuillan (1999) showed by infrared spectroscopy that phosphate ions adsorb strongly to $TiO_2$ through the formation of surface complexes. However, the interaction of phosphates with metal oxides is governed by several factors, in particular pH. Kang et al. (2011) showed through nuclear magnetic resonance (NMR) spectroscopy that at pH values between 4.5 - 7 the sorption of phosphates to $TiO_2$ surface is almost irreversible, but at pH 9 an initial reversibility is followed by the re-adsorption and stabilization of phosphates on $TiO_2$ surface. Based on these studies it was shown that some nanoparticles (especially metal oxides) have the ability to adsorb and/or precipitate P forms in aqueous solution. Thus, it is expected that nano-$TiO_2$ has contributed for the reduction in phosphates observed after its addition to the PT microcosms. However, the high pH recorded on the microcosms may have contributed for lowering the efficiency of this nanomaterial in the control of phosphate release from the sediment.

In our study, nano-$TiO_2$ was also effective, at least in part, in reducing total N content, both before the second addition of phytoplankton to the microcosms (throughout the days 32 and 36, the values varied from 76.5 mg $L^{-1}$ to 61.6 mg $L^{-1}$), but also until the end of the experiment (57.0 mg $L^{-1}$ at day 64). However, the possible loss of N from the system by the mechanisms described in the previous section cannot be discarded. Recently, many treatment approaches based on the unique physico-chemical properties of nanomaterials have been introduced and developed in order to remove N from wastewater. Similarly to our results, Zheng et al. (2011) observed that nano-$TiO_2$ (pure anatase, 25 nm) at concentrations lower than those used in our study (50 mg $L^{-1}$) effectively reduced the total N loading, both under anaerobic and aerobic conditions and during a long period of exposure (70 days). Also, nano-$Al_2O_3$ (particle size of 20 ± 5 nm, at 50 mg $L^{-1}$) was effective in reducing total N in wastewater (Chen et al., 2012). The photocatalytic activity of nano-$TiO_2$ may also have contributed for degrading organic forms of nutrients, as well as for inhibiting the biological productivity in the microcosms, especially at PT (for the reasons herein described as the likely long persistence of the NM in the water column in these microcosms). As far as nano-$TiO_2$ photocatalytic activity is concerned, several studies have reported its effectiveness in water treatment, because of its biological and chemical stability and favourable band-gap energy (Gaya and Abdullah,

2008; Kwon et al., 2008; Teh and Mohamed, 2011). Under visible light illumination, $TiO_2$ photocatalytic treatment seemed to be effective in water disinfection, particularly in the degradation of organic molecules such as cyanotoxins (Fagan et al., 2015; Lawton et al., 2003; Wang et al., 2015). Based on this capacity, it was considered that nanomaterials could be the ultimate solution in the prevention of cyanobacterial blooms in natural water eutrophication (Matthijs et al., 2016).

After the addition of nano-$TiO_2$ to PT microcosms, pH started to decrease until day 43, followed by a stabilization up to day 64. This pH decrease and the subsequent stabilization could have been related with shifts on dominant algae inside the microcosms. Algae may raise the pH at night by the release of $CO_2$ during respiration, or whenever they are below their compensation level (Dubinsky and Rotem, 1974). This could create the condition for the dominance of other groups of algae and subsequent pH stabilization. Such shifts in the phytoplankton community may also have contributed for the dominance of certain species with a greater ability to produce and release organic compounds to deal with stressful conditions (e.g. unfavourable pH), thus causing an increase in conductivity as was observed for all the treatments (Villacorte et al., 2015) On day 31, conductivity measurements were made in PT microcosms before the addition of nano-$TiO_2$. Thus, the rise of this parameter in this treatment cannot be caused by the addition of the nanopowder.

In a general way, nano-$TiO_2$ seemed to be responsible for a short-term reduction of phosphates in the overlying water of PT microcosms. In contrast, the reduction of N compounds decreased progressively until the end of the experiment. However, although changes on nutrients balance were not sufficient to suppress phytoplankton productivity, they were sufficient to keep the biological productivity below the values recorded on CTL2, as well as to prevent the enhanced development of phytoplankton caused by the second inoculation on day 36.

The chronic assay with *D. magna* showed a reduction in the average number of neonates and in the somatic growth of adults, as well as the intrinsic growth rate of population (*r*). However, reproduction was the most sensitive endpoint compared to all the other parameters considered. According to $OD_{440nm}$ variation, the phytoplankton

productivity was reduced in the PT treatment in comparison with CTL2 [(day 43 – day 64): PT = 0.081 ± 0.023 – 0.046 ± 0.016; CTL2 = 0.105 ± 0.034 – 0.083 ± 0.027], but was kept stable in the last days of the experiment. For that reason, both the limitation of food, as well as the quality of food, may have been responsible for the negative responses obtained. According to other investigations, nano-$TiO_2$ easily forms aggregates that rapidly settle, when suspended in aqueous media (Campos et al., 2013; Chowdhury et al., 2013; Lopes et al., 2012; Metzler et al., 2011; Tiede et al., 2009). Campos et al. (2013), testing different types of $TiO_2$ nanoparticles (including P-25), concluded that all were able to reduce algal concentration in water, especially in the highest concentration of the nanoparticles (10 mg $L^{-1}$) due to the formation of large $TiO_2$-algae agglomerates that rapidly settled, depleting dramatically the food available for *D. magna* and impairing significantly reproduction and fitness of these organisms. However, at high algal densities, only P-25 $TiO_2$ caused similar effects on daphnids, both under semi-static and particle re-suspension conditions. This lead these authors to conclude that in this case food acquisition was compromised by gut clogging. Li et al. (2011) also demonstrated that both $TiO_2$ and $Al_2O_3$, at concentrations between 10 and 100 mg $L^{-1}$, interacted with algal cells forming large aggregates that caused food depletion to *Ceriodaphnia dubia* mediating responses to both nanomaterials.

Nevertheless, the whitish colour of the water, in the preventive treatment microcosms, after nano-$TiO_2$ application, led us to suspect that at least some small aggregates or particles, may have persisted for more time in suspension, especially in the first centimetres of the water column. According to some authors, absorption of nano-$TiO_2$ on algal cell surface and the subsequent block of electrolyte and metabolite transport across the membrane was suggested as the primary toxicity mechanism at lower trophic levels (Metzler et al., 2012; Rodríguez-González et al., 2010). In this context, several studies demonstrated the ability of *D. magna* to directly incorporate nano-$TiO_2$ agglomerated with algal cells by dietary exposure (De Schamphelaere et al., 2007, 2004; Evens et al., 2009; Liu et al., 2002) or by passive diffusion of small particles that can be taken from the surrounding water (Pane et al. 2004; Mansfield et al. 2014). Comprising of both chronic toxicity and bioaccumulation potential of nano-$TiO_2$ in the daphnids,

Fouqueray et al. (2012) performed one of the few studies testing nano-$TiO_2$ bioavailability taken via food uptake. They chronically fed *D. magna* with *R. subcapitata* contaminated with $TiO_2$ residues (50-700 nm in size), at concentrations from 0.1 to 10 mg $L^{-1}$, and a significant decrease on growth and reproduction was observed. On the other hand, regarding to waterborne exposures, Zhu et al. (2010) tested nano-$TiO_2$ (21 nm) dispersed in the medium of *Daphnia*, and also observed a severe growth retardation and mortality. Further, changes in reproductive output were detected on organisms chronically exposed to 5 mg $L^{-1}$. In our study, a high concentration was added to PT microcosms (1 g $L^{-1}$), thus it is probable that the concentrations in water have decreased to levels as low as those reported by these authors after aggregation and settlement. According to Zhu et al. (2010) and Rosenkranz et al. (2009), the adverse effects observed in the reproduction of *D. magna* were primarily related to the uptake of nanoparticles in their gut that might reduce the amount of algae consumed, disturbing their energy budget allocation. This fact may link changes in the energy budget with increasing metabolic costs and/or a reduction of nutrient assimilation (De Schamphelaere et al., 2007; Evens et al., 2009). Similar results were also obtained by Nogueira et al. (2015a), who observed an increasing sensitivity of *D. magna* to nano-$TiO_2$ (100% anatase; < 25 nm) throughout the exposure time: when the organisms exposed for 21 days (at concentrations from 8.19 to 20 mg $L^{-1}$), a significant reduction in the number of neonates and in the intrinsic growth rate of population (at 16 mg $L^{-1}$) was seen in time. Moreover, there are some studies addressing the potential photoreactivity and increase in toxicity of nano-$TiO_2$ for *D. magna* in the presence of artificial sunlight. In this context, Hund-Rinke and Simon (2006) used simulated sunlight (300-800 nm) to pre-irradiate nano-$TiO_2$ particles (100% anatase; < 25 nm), before testing their acute toxicity with *D. magna*. The authors reported that *D. magna* immobilization seemed to increase with photocatalytic activation of the particles, since 73% of test organisms were immobilized when exposed to a concentration of 2.5 mg $TiO_2$ $L^{-1}$. In the tests with non-illuminated products, 28% of the daphnids were immobilized when exposed to the same concentration. Moreover, in aquatic assays done at environmentally realistic levels of solar irradiation, Ma et al. (2012a, 2012b) reported that the acute toxicity of nano-$TiO_2$ for *D. magna* was increased by four orders of

magnitude by irradiation. These authors attribute this enhanced toxic effect to the generation of reactive oxygen species (ROS) by the photocatalytic activity of the nanomaterial. In contrast, Wiench et al. (2009) reported that the pre-treatment of nano-$TiO_2$ with artificial sunlight irradiation (for 30 min at 300-800 nm) caused no increase in acute toxicity for daphnids, by comparing with the same non-irradiated material. In summary, and considering that the aggregation of nano-$TiO_2$ with algae and its subsequent settlement was likely less pronounced in PT microcosms rather than in RT microcosms (see Section 4.4.3), as demonstrated by the decrease of $OD_{440nm}$ values in both treatments, the ability of light to increase the toxicity of nano-$TiO_2$ may have also been responsible for the most pronounced effects on daphnid endpoints recorded at PT microcosms. As highlighted by Campos et al. (2013), effects of the nanomaterial on growth and reproduction of daphnids are likely caused by different but simultaneous processes, namely direct toxicity, food depletion, and decreased food intake due to gut clogging or due to partially ingestion of nanomaterials that are not edible and which are adsorbed to algal cells.

The significant effects of nano-$TiO_2$ on *Lemna minor* growth were only observed in plants from PT, with a significant reduction in the number of fronds both at 7 days and 21 days of exposure in comparison with control (CTL2) values. Pinheiro et al. (2013) exposed *Lemna* to nano-$TiO_2$ (average particle size of 28 ± 11 nm; concentrations of 6.25 and 25 mg $L^{-1}$), and using nuclear microscopy observed an irregular deposition of titanium in the epidermis of the fronds and roots, while no internalization was recorded. Kim et al. (2011), working with a high range of nano-$TiO_2$ (size of 2-3 nm) concentrations, observed no statistical differences in specific growth rate of *Lemna* at 100 mg $L^{-1}$, whereas a significant reduction (about 51%) was observed in plants exposed to 500 mg $L^{-1}$. In accordance with our results, these studies also mentioned morphological alterations in *Lemna* fronds, namely chlorosis (a progression of green to yellow on the frond), in nano-$TiO_2$ exposures at 25 mg $L^{-1}$ (Pinheiro et al., 2013) and 250 mg $L^{-1}$ (Kim et al., 2011). Also, Song et al. (2012) tested nano-$TiO_2$ (5-10 nm of size) effect on *L. minor* growth rate and observed a significant inhibition at concentrations of 200, 500, 1000 and 2000 mg $L^{-1}$, in a concentration-dependent way. Therefore, if nanoparticles have a longer residence time in

aquatic environment, it is expected that plants could interact with them more efficiently, with changes on growth and development parameters (Capaldi Arruda et al., 2015; Navarro et al., 2008).

Finally, no growth inhibition was detected in *C. riparius* during the experiments. One possible explanation was that nano-$TiO_2$ persisted for a long time in the water column, and only large aggregates had settled in the sediment, as suggested by the more pronounced effects on *D. magna* and *L. minor* species. Similar to our results, Lee et al. (2009) reported neither mortality nor alteration in the growth of *C. riparius* exposed to nano-$TiO_2$ (7 and 20 nm, at 1 mg $L^{-1}$). However, we expect that higher concentrations were attained the surface of the sediment, as nano-$TiO_2$ was added precisely to work as a capping agent. Recently, Nogueira et al. (2015b) tested the impacts of NP solid residues (SRs), from the treatment of wastewaters (kraft pulp mill, olive oil mill, and mine effluents), on the development of a sediment-dwelling organism, namely *C. riparius*. According to these authors, a significant decrease in body length of chironomids exposed to kraft paper mill effluent SR was apparently caused by the organic compounds bound to $TiO_2$ powder (1 g $L^{-1}$) added to treat the effluent, rather than by the nanomaterial.

### 4.4.3. Remediation treatment efficiency and ecotoxicological impacts on biota

The remediation treatment started immediately after a high algal density had been established by a second inoculation of microcosms with algae from the lake. The addition of nano-$TiO_2$ immediately started a reduction in $OD_{440nm}$ values persisting until day 64. Total P also showed a decreasing tendency during this period. However, the P levels in RT microcosms were always higher than those recorded on CTL2. In accordance with the results from the preventive treatment, the application of nano-$TiO_2$ in RT was also effective in reducing total N and phosphate content, and the explanations were in agreement to those mentioned in the previous treatment, as far as the microcosms conditions (e.g., pH, conductivity, dissolved oxygen) remained stable (Supplementary material, Table SIV.1). Moreover, the RT was effective in reducing the phytoplankton growth, particularly during the *algal bloom* episode ($OD_{440nm}$: from 0.159 to 0.023). As discussed in the previous section, and in line with our results are the findings from

Metzler et al. (2011), Campos et al. (2013) and Chowdhury et al. (2013), whose experiments reported nano-$TiO_2$ ability to form aggregates with algal cells forming clusters that were removed from the overlying water. Furthermore, it was also shown that large nano-$TiO_2$ aggregates could entrap algal cells, reducing light availability and inhibiting growth in the water column (Aruoja et al. 2009).

In fact, the *D. magna* results showed a significant reduction in the number of neonates and in the intrinsic growth rate of population (*r*) in the RT microcosms, while no significant inhibitory effects were observed in the somatic growth rate and in the number of broods. In our opinion, and as previously mentioned, these results might be a primary response of limited food conditions to which daphnids were exposed in RT water column, due to the interaction of nano-$TiO_2$ with algal cells. According to Campos et al. (2013) the reduction of food availability by nano-$TiO_2$ may occur at different food levels, thus meaning that this mechanism is a possible explanation for both PT and RT treatments. Such food limitation affected first the reproduction and then the growth of daphnids. In fact, the formation of aggregates in natural waters using nano-$TiO_2$ is strongly linked with high ionic content and high pH levels (Sillanpää et al., 2011), conditions that were observed inside RT microcosms (Conductivity $_{max\ value}$ = 1106 µS $cm^{-1}$ ; pH $_{max\ value}$ = 8.68) and which have likely enhanced the aggregation between nano-$TiO_2$ and algal cells, both in PT and RT microcosms.

The highest abundance of algae in the RT microcosms, when nano-$TiO_2$ was applied, may have contributed for forming large $TiO_2$-algae aggregates removing more nanomaterial from the water column making it less available to cause toxic effects on daphnids and on aquatic plants. Therefore, with respect to *L. minor* growth inhibition test, no alterations were observed in the number of fronds growing in RT compared to CTL2, after 21 days of exposure. Interestingly, and in contrast to studies reporting negative effects, and described above, current literature also reports no adverse effect on the growth rate of *L. minor* at 5 mg $L^{-1}$ (Li et al., 2013) and at concentrations up to 90 mg $L^{-1}$ (Picado et al., 2015) of nano-$TiO_2$. Additionally, some other studies reported the positive effects of nano-$TiO_2$ to the plants, namely the induction of seed germination, plant growth and the increase of dry weight (Feizi et al., 2013; Zheng et al., 2005),

probably by the influence of titanium on the uptake of other essential elements by roots (Dumon and Ernst, 1988).

The results related to *C. riparius* growth inhibition test revealed a significant reduction in the growth of organisms exposed in the RT microcosms. In fact, since $TiO_2$-algae agglomerates settled in the bottom sediment of RT and considering the significant sediment reworking activity of *C. riparius* (Bour et al., 2014), we assume that the ingestion of contaminated sediment or direct ingestion of nanoparticles are very likely to occur, thereby enhancing the risk to benthic organisms. In accordance with our results, Oberholster et al. (2011) reported a significant inhibition of *Chironomus* larvae length growth in comparison to control organisms in a concentration-dependent manner over the 10-day exposure period to seven different nanoparticles. These authors pointed out that the decrease of this sublethal parameter was likely due to toxic effects NPs, which caused a decrease in cellular energy (ATP levels) available, influencing the switch between apoptotic and necrotic cells (Sweet et al., 1999). In fact, few studies have focused on the impacts of nano-$TiO_2$ in *C. riparius* endpoints, in comparison to those reporting these effects after exposure to other nanomaterials.

Since *D. magna* and *L. minor* results presented less pronounced effects in RT in comparison with those in PT, we suppose that nano-$TiO_2$ in the RT was likely more available for sediment organisms than for those in the water column, thus explaining the more pronounced effects on *C. riparius* than on the other organisms observed in these microcosms. From the results obtained, it appears that the more pronounced availability of nano-$TiO_2$ for sediment-dwelling organisms might be related to the settlement of larger aggregates formed between nano-$TiO_2$ and algae during the *algal bloom* episodes.

### 4.5. Conclusions

Nutrient reduction, as a part of a lake management plan, needs to be a highly customized process. Thus, considering a chemical approach, the PT was effective in controlling the release of phosphate from the upper layer of sediment and in reducing phosphate and subsequent total P content in the water column. Furthermore, nano-$TiO_2$ treatment revealed to be effective in reducing total N in the water column, although the role of

denitrification processes on N release from microcosms cannot be neglected. The changes on nutrient dynamics contributed for controlling phytoplankton growth, which occurred in a more progressive way until the end of the experiment. Nevertheless, the toxic effects on pelagic organisms were more pronounced in the presence of nano-$TiO_2$ than in its absence. Such toxic effects were likely multifactorial, but probably the longer persistence of the NM in the water column, left room for a great interaction of *D. magna* and of *L. minor* with the nanomaterial, the toxicity of which could also have been enhanced by light. In a real scenario, the NM could be added close to sediment, as it was made in other remediation projects (Grieger et al., 2010; Karn et al., 2009; Kim et al., 2009; Kurniawan et al., 2012; Lewis et al., 2016) to reduce the negative effects on limnetic species, but this will not prevent the impacts on benthic species.

The results from the RT microcosms showed that nano-$TiO_2$ could be effective in reducing algal blooms, since the abrupt reduction in $OD_{440nm}$ values demonstrated that a more pronounced nano-$TiO_2$ interaction with algae has likely occurred than otherwise, enhancing the formation of aggregates and their rapid settlement in sediment. This conclusion is also corroborated by the less pronounced toxic effects observed for pelagic species, in contrast to chironomids that were significantly more affected by this treatment.

Although our results provided clear evidence that the control of algal blooms could be attained with nano-$TiO_2$ , with both preventive and remediation approaches, the nanomaterial may have impacts on the biota of the system. Thus, future efforts should be focused in testing other nanomaterials for their effectiveness in preventing or mitigating algal blooms on shallow lakes through an integrated approach, also aimed at assessing their ecological impacts on species from eutrophic scenarios. Finally, since the formulation of nano-$TiO_2$ used in this experiment seemed to have deleterious effects for non-target species, the application of nano-$TiO_2$ with appropriate coating materials could be considered (Ochiai et al., 2010), as well as the fixation of nanoparticles in supporting materials that could be easily placed and removed from these systems, without compromising the chemical quality of the water and of the sediments. Further, functionalization, or the use of other nanomaterials with a higher adsorption capacity

may allow a reduction of the amount of nanomaterial needed, contributing to both more cost-effective and less impactful treatment approaches. The effectiveness of these treatments for more extended periods needs also to be analysed.

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**This article contains Supplementary material.**

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## 4.7. Supplementary material

**Table SIV.1.** Variation of dissolved oxygen concentration (mg $L^{-1}$) in the water column of the microcosms during the stabilization period, preventive treatment (PT) and the remediation treatment (RT). Values are the means±standard deviation.

| | Stabilization Period | | | | Preventive Treatment (PT) | | | |
|---|---|---|---|---|---|---|---|---|
| | | | | | | Remediation Treatment (RT) | | |
| **Dissolved oxygen (mg $L^{-1}$)** | **Day 1** | **Day 10** | **Day 20** | **Day 30** | **Day 36** | **Day 43** | **Day 53** | **Day 64** |
| **CTL1** | 9.93±0.02 | 9.79±0.07 | 9.00±0.12 | 9.13±0.03 | 8.95±0.04 | 8.83 ± 0.03 | 8.88 ± 0.02 | 8.86 ± 0.03 |
| **CTL2** | 9.82±0.20 | 9.36±0.02 | 9.42±0.02 | 9.15±0.16 | 9.01±0.03 | 8.96 ± 0.03 | 8.90 ± 0.03 | 8.83 ± 0.06 |
| **PT** | 9.81±0.20 | 9.36±0.02 | 9.42±0.03 | 9.25±0.04 | 9.09±0.06 | 8.96 ± 0.03 | 8.77 ± 0.10 | 8.78 ± 0.04 |
| **RT** | 9.93±0.04 | 9.41±0.05 | 9.38±0.04 | 9.19±0.08 | 8.87±0.05 | 8.53 ± 0.13 | 8.61 ± 0.24 | 8.65 ± 0.07 |

**Table SIV.2.** One-way and two-way ANOVA's summary (df, F ratio and p value) and post-hoc Tukey multicomparison test results, regarding nutrients content and $OD_{440nm}$ values during the stabilization period, preventive treatment (PT) and remediation treatment (RT).

| | | | Two-way ANOVA | | | |
|---|---|---|---|---|---|---|
| | **Endpoint** | **Source** | **df** | **F ratio** | ***p* value** | **One-way ANOVA and Tukey multiple comparisons** |
| **Stabilization Period** | Total P | Day | 1,073 | 1.871 | 0.263 | |
| | | Day*Treatment | 2,146 | 0.47 | 0.673 | |
| | | Treatment | 2 | 0.02 | 0.98 | |
| | Total N | Day | 2,447 | 98.617 | <0.001 | Day 1≠Days 10,20,30;Day 20≠Day 10, Day 30 |
| | | Day*Treatment | 4,895 | 2.158 | 0.116 | |
| | | Treatment | 2 | 1.381 | 0.321 | |
| | Phosphates | Day | 1,501 | 53.414 | <0.001 | Day 1[(F=20;p<0.001):CTL1≠CTL2,PT,RT]≠ ≠Day 10[(F=3.644;*p*<0.05):CTL2≠RT] |
| | | Day*Treatment | 4,502 | 1.715 | 0.19 | |
| | | Treatment | 3 | 7.077 | 0.006 | |
| | Nitrates | Day | 2,867 | 13.588 | <0.001 | Day 1≠Day 20, Day 30; Day 10≠Day 2 |
| | | Day*Treatment | 8,601 | 1.808 | 0.113 | |
| | | Treatment | 3 | 2.858 | 0.091 | |
| | $OD_{440nm}$ | Day | 1,14 | 20.456 | <0.001 | Day 1≠Day 20, Day 30 |
| | | Day*Treatment | 3,42 | 0.792 | 0.511 | |
| | | Treatment | 1,3 | 4.275 | 0.052 | |

| | | One-way ANOVA | | | |
|---|---|---|---|---|---|
| | **Endpoint** | **df** | **F ratio** | ***p* value** | **Tukey multiple comparisons** |
| **Preventive Treatment (PT)** | P total | 2,9 | 9.707 | 0.006 | Day 32 ≠ Day 40; Day 40 ≠ Day 64 |
| | N total | 2,9 | 12.174 | 0.003 | Day 32 ≠ Day 40; Dia 32 ≠ Day 64 |
| | Phosphates | 2,8 | 0.466 | 0.644 | |
| | Nitrates | 2,9 | 1.167 | 0.354 | |
| | $OD_{440nm}$ | 2,9 | 1.409 | 0.294 | |

**Table SIV.2.** (continued)

| | | | Two-way ANOVA | | | |
|---|---|---|---|---|---|---|
| | **Endpoint** | **Source** | **df** | **F ratio** | ***p* value** | **One-way ANOVA and Tukey multiple comparisons** |
| **Remediation Treatment (RT)** | Total P | Day | 1,212 | 2.744 | 0.122 | Day 49 [(F=8.043; p=0.010): RT ≠ CTL2, PT] ≠ ≠ Day 64 [(F=12.599; *p*=0.002): RT ≠ CTL2, PT] |
| | | Day*Treatment | 2,424 | 2.954 | 0.088 | |
| | | Treatment | 2 | 8.678 | 0.008 | RT≠CTL2,PT |
| | Total N | Day | 1,748 | 7.652 | 0.006 | Day 43 [( F=7.634; *p*=0.012): PT≠RT] ≠ Day 49, Day 64 |
| | | Day*Treatment | 3,496 | 4.865 | 0.011 | |
| | | Treatment | 2 | 2.976 | 0.102 | |
| | Phosphates | Day | 1,215 | 7.648 | 0.011 | Day 64 ≠ Day 43, Day 49 |
| | | Day*Treatment | 3,644 | 0.911 | 0.476 | |
| | | Treatment | 3 | 1.589 | 0.244 | |
| | Nitrates | Day | 1,193 | 1.222 | 0.298 | |
| | | Day*Treatment | 3,578 | 1.932 | 0.164 | |
| | | Treatment | 3 | 7.29 | 0.005 | CTL1 ≠ CTL2, RT; PT ≠ CTL2, RT |
| | $OD_{440nm}$ | Day | 1,439 | 195.978 | <0.001 | Day 43(F=22.424;*p*<0.001): CTL2≠CTL2,PT,RT; RT≠CTL2,PT]≠ ≠Day49[(F=10.382;*p*=0.001):CTL1≠CTL2,PT,RT]≠ ≠Day 64[(F=13.040;*p*<0.001):CTL2≠CTL1,PT,RT] |
| | | Day*Treatment | 4,316 | 64.748 | <0.001 | |
| | | Treatment | 3 | 11.703 | 0.001 | CTL1≠CTL2, PT, RT |

# Chapter V

## Conclusions and final remarks

## 5.1. Conclusions and final remarks

Lakes are critical in the provision of many services for human welfare, for freshwater diversity and also for the ecosystem services, and densely populated areas usually surround them (Jogo and Hassan, 2010; López-Merino et al., 2011; Namaalwa et al., 2013). Severe contamination has occurred in many lakes around the world, and some of these lakes have experienced algal blooms, which have directly resulted in the loss of several ecosystem services provided by these freshwater ecosystems, including the provision of water for human consumption (Kuiper et al., 2015; Zhang et al., 2015). Diffuse pollution from agriculture has been one of the major contributors of nutrients and of contaminants in the adjacent water bodies, especially by the runoff of manure, chemical fertilisers and/or plant protection products intentionally applied to agricultural soils (Horton et al., 2017; Schmidt Rivera et al., 2017; Shappell et al., 2016). Through leaching or surface and sub-surface runoff, the nutrients and contaminants from soils can enter the water bodies (Bromilow et al., 2006; Hooda et al., 2000), and may compromise their ecological integrity. In this way, the interactions between aquatic and terrestrial compartments should be evaluated, not only to predict the environmental risks of such complex interactions, but also to determine the direct and indirect effects on the aquatic species exposed to toxic levels of agricultural contaminants. In this context, the present work aimed to contribute for the increase of knowledge about the impact of diffuse pollution from agriculture to aquatic species, and also the ecological risk assessment (ERA) of an area undergoing diffuse pollution from agriculture activities, based on a combined procedure of ecotoxicological and chemical analysis, for a soil screening (tier 1) of the ERA framework. Finally, our study also aimed to assess the application of environmental measures in the remediation of shallow freshwater bodies exposed to that type of contamination, based on nanotechnology.

Taking this in consideration, our first approach aimed to study the indirect effects of diffuse pollution, namely how the exposure to the herbicide pendimethalin (Prowl®), under environmentally relevant concentrations, may compromise the nutritional composition of food for daphnids, a relevant group of primary consumers of freshwater food webs, thus affecting their reproduction performance and subsequently the long

term sustainability of active populations of this grazer, which has been reported as being able to control phytoplankton blooms (Peretyatko et al., 2012, 2009). The nutritional quality of food was assessed and algae exposed to the herbicide were analysed for protein, carbohydrate and fatty acid content. The chemical composition of *Raphidocelis subcapitata* growth in a medium enriched with pendimethalin (in a concentration equivalent to the $EC_{20}$ for growth inhibition for algae) revealed a slight decrease in fatty acids levels, with a particular decrease of essential ω9 monounsaturated fatty acids. However, the protein content was high in algae grown in the presence of pendimethalin, but no significant differences were recorded for carbohydrates content caused by the exposure of the pesticide. The reproduction of *Daphnia magna* was significantly reduced in organisms fed with contaminated algae, but no significant differences were recorded at the time to first broad, brood size and *D. magna* growth rate between daphnids fed with contaminated and non-contaminated algae. Additionally, an internal pendimethalin body burden of 4.226 $\mu g\ g^{-1}$ was accumulated by daphnids fed with algae exposed to the pesticide. In general, the reported results suggest that the uptake of pendimethalin through food, at low concentrations of Prowl® that could be found under realistic scenarios in agricultural catchment, was clearly responsible for the bioaccumulation of this pesticide and for changes on the reproductive output of *D. magna*. Furthermore, the application of this type of herbicides may affect, direct and/or indirectly, the zooplanktonic community compromising the system ability in responding to the nutrient enrichment. Overall, this study underlines the importance of considering both direct and indirect effects of pesticides (e.g. low quality food or bioaccumulation via food uptake) on key freshwater aquatic species, thus increasing the realism of environmental risk assessment (ERA) evaluations.

Considering the previous results and the need to diagnose the hazard of the available fraction of contaminants in soils, and its impact on non-target organisms of different trophic levels, our study aimed to provide more deep insights to the ERA previously started to an area undergoing diffuse pollution by plant protection products used in agriculture in a drainage area surrounding a lacustrine ecosystem in the Centre of Portugal, namely Lake Vela. Previous results of ERA for Lake Vela confirmed the

contamination of water and sediments with the pesticides more applied in the area (Abrantes et al., 2010, 2009, 2006), and also concluded that the use of pesticides in the lands near Lake Vela, combined with some particular properties of local soils, can contribute to the contamination of surface and groundwater resources (Abrantes et al., 2008). In this context, and in order to support these findings and increase the knowledge about the potential effects of soils in that particular eutrophic ecosystem, our approach aimed to perform a soil risk assessment of the ERA framework by combining ecotoxicological tests and chemical analysis of organic contaminants to assess contaminated soils of that extensive area impacted by diffuse pollution. Moreover, we intended to propose a new approach for tier 1, in which the ecotoxicological line of evidence (EcotoxLoE) selected the soils for chemical analysis of potential contaminants and a subsequent evaluation of integrated risks was performed considering both the chemical line of evidence (ChemLoE) and the EcotoxLoE. Using a low cost-effective and time-effective battery of bioassays, the results confirmed that the application of EcotoxLoE (tier 1) seemed to be effective in evaluating the toxicity of soils from Lake Vela drainage basin. Furthermore, the chemical data reinforces the conclusions made based on the EcotoxLoE, highlighting the role of soil organisms as more sensitive indicators of the effects of mixtures of soil contaminants. Considering that analytical methods are extremely expensive and that their use could limit the application of the ERA framework, on a routine basis, to areas under diffuse pollution, the approach proposed in our study can provide a feasible evaluation for tier 1, with lower costs. Thus, economic constraints should not be the reason to impede the assessment of the risks of pesticides in the drainage basin of small freshwater ecosystems.

Besides the recognized contamination with pesticides, this freshwater resource has also been impacted by the input of nutrients mainly by plant protection products used in agriculture, which has accelerated the eutrophication process in the lake. In this context, we evaluated the effectiveness of the nanomaterial $TiO_2$ in the remediation of eutrophic waters, both for reducing nutrient desorption and release from sediments (preventive treatment – PT) and for eliminating algal blooms (remediation treatment – RT). Moreover, we also assessed the potential impacts of nano-$TiO_2$ application on key

species from shallow freshwater lakes, in order to support the safety of future applications. The results showed the effectiveness of nano-$TiO_2$ in controlling the release of phosphates from surface sediment and the subsequent reduction of total phosphorus in the water column. Total nitrogen concentration was also reduced. Nano-$TiO_2$ revealed ability in interacts with algal cells, mainly in RT, enhancing the formation of aggregates and their rapid settlement, thus reducing the algal blooms. In general, both treatments caused deleterious effects on freshwater species, in which *Daphnia magna* and *Lemna minor* showed a significant inhibition in several endpoints in PT, while no inhibitory effect on the growth of *Chironomus riparius* was recorded in that treatment. However, *C. riparius* was the most affected species in RT, probably caused by the formation of larger $TiO_2$-algae aggregates, under a high algal density that rapidly settle in the sediment and become less available for pelagic species. Although results provided clear evidence that the control of internal nutrient loading and the mitigation of algal blooms could be attained with nano-$TiO_2$ in both treatments, their negative effects on the biota of the system have to be seriously considered.

This study has also shown that the impacts of diffuse pollution both in water and soils should be integrated into the application of the ecological risk assessment framework, combining ecotoxicological tests and chemical analysis to assess contaminated lands. Further, the present work proposed a new approach for tier 1 for the soil risk assessment, with a slight modification of the scheme proposed by Jensen and Mesman (2006), in which the ecotoxicological tests are performed first for selecting the samples for subsequent chemical analysis. However, when internal and external sources of nutrients are the main drivers of the ecosystem variability, maintaining or promoting the eutrophication phenomena, several remediation strategies should be considered to face the eutrophication problem. Since nanomaterials can be applied to water remediation with promising results, the present work had recognized the effectiveness of the nano-$TiO_2$ in controlling the internal loading of nutrients and for eliminating algal blooms, while negatively affect non-target species of eutrophic systems. Thus, future efforts should be focused in testing different eco-friendly nanomaterials (such as

nanoclays and nanosilica) for their effectiveness in preventing or mitigating algal blooms in shallow lakes through an integrated approach, similar to the one tested in our study.

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